Investigating the roles of *MSS51* in translation activation of *COX1* and assembly of Cox1p into cytochrome *c* oxidase

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Investigating the roles of *MSS51* in translation activation of *COX1* and assembly of Cox1p into cytochrome *c* oxidase

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The *S. cerevisiae* the nuclear gene *MSS51* is required for synthesis of mitochondrially encoded Cox1, the largest subunit of cytochrome *c* oxidase. *MSS51* functionally interacts with the 5'-untranslated region (UTR) of *COX1* mRNA to activate translation, and also physically interacts with newly synthesized Cox1p as a component of early assembly intermediates. These dual functions of Mss51 couple Cox1 synthesis to assembly of cytochrome *c* oxidase. Mss51 has no previously described amino acid sequence domains or motifs.

Two partial separation-of-function *mss51* alleles were obtained by screening for mutations that allow expression of the *ARG8^m* reporter inserted in place of the *COX1* coding sequence between the *COX1* UTRs, but not the *COX1* coding sequence flanked by UTRs from the *COX2* mRNA. One, *mss51-W64R,* also fails to express *COX1* from its native locus. Thus, *mss51-W64R* activates translation at the *COX1* 5' UTR, but has a defect in downstream assembly steps of Cox1. When overexpressed, *mss51-W64R* was able to promote respiratory growth, indicating that *mss51-W64R* is not completely deficient in assembly functions. I therefore propose that the single missense mutation of *mss51-W64R* is integral in its role of formation of the Cox1 assembly complex.

A second allele identified in the screen, *mss51-102,* allows only weak respiratory growth when *COX1* is flanked by *COX2* UTRs, but strong respiratory growth when *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence. I selected for spontaneous mutants that enhanced the weak respiration in a strain with *mss51-102* bearing *cox2::COX1 cox1::ARG8m* mtDNA. I identified a novel mitochondrial rearrangement resulting from the apparent duplication of the *COX1* 5' leader, causing it to be present at both *COX1* and *ARG8m.* I propose that the strong preference of *mss51-102* for the *COX1* 5' leader to be present in *cis* to the coding sequence implies that the same Mss51p molecule that activates translation is transferred in *cis* to the nascent Cox1 peptide.

BIOGRAPHICAL SKETCH

Zach Via was born in Fairbanks, Alaska in June of 1984 and grew up in the community of Ester, Alaska. His early interests involved taking advantage of Alaska's many wilderness opportunities, and became an avid and competitive cross country skier in high school. While in high school, Zach enrolled in an excellent biotechnology course taught by Mr. Don Peterson, which started an intense interest in science.

Zach enrolled in the University of New Hampshire in the fall of 2002 as an undeclared liberal arts major, but after taking his first introductory biology course from Dr. Charles Walker (Ph.D. Cornell '76), Zach quickly declared as a major in molecular and cellular development, and Dr. Walker became Zach's advisor. Zach had the opportunity to work in the Walker lab, studying the conservation of light sensing genes in sea urchins. The project was an excellent learning opportunity and an introduction to the field of independent research. Largely on the advice of Dr. Walker (both concerning the science and the quality of the dry Rieslings) Zach enrolled in his doctoral research at Cornell in the fall of 2006 in the Field of Biochemistry, Molecular and Cellular Biology. During his rotation projects at Cornell, Zach had the opportunity to work on three intellectually stimulating projects in varied organisms, studying the regulation of *Greatwall* gene in *Xenopus laevis* in the lab of Dr. Michael Goldberg, a mutant screen involving *MSS51* in *Saccharomyces cerevisiae* in Dr. Thomas Fox's lab, and a large screen of transcription factors in *Caenorhabditis elegans* in the lab of Dr. Kelly Liu.

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LITERATURE REVIEW

Introduction

Mitochondria are involved in a host of cellular functions; the most well known is energy production via the electron transport chain, but mitochondria are also involved in other metabolic activities essential to the cell, including Fe/S cluster formation and apoptosis (Reichert and Neupert 2004). Mitochondria contain a distinct genome, evolutionarily related to an alpha-proteobacterium and likely derived from an endosymbiotic event (Yang, Oyaizu et al. 1985; Huynen, de Hollander et al. 2009). It is remarkable in *Saccharomyces cerevisiae* that the mitochondrial DNA encodes only 8 protein coding genes, yet approximately 250 nuclear encoded protein products are targeted to the mitochondria for proper regulation, translation and assembly of those genes (Herrmann and Neupert 2000). The complex interplay between nuclear- and mitochondrial-encoded proteins is of biological interest, as proper function of the mitochondria is integral to the viability of an organism. Mitochondrial genes are inherited in a non-Mendelian fashion; in yeast mitochondria are inherited in a biparental fashion and eventually will undergo mitotic segregation into pure types, while in mammals mitochondrial genes are often inherited maternally.

The *S. cerevisiae* mitochondrial genome is approximately 86 kb, and encodes 8 major protein coding genes, 2 ribosomal RNAs and 24 transfer RNAs (Foury, Roganti et al. 1998). The human mitochondrial genome is smaller at 16.5 kb, and

encodes 24 tRNAs, 2 rRNAs and 13 protein-coding genes (Anderson, Bankier et al. 1981). *S. cerevisiae* is an excellent organism in which to study the translation of mitochondrial genes and the interaction between nuclear and mitochondrial polypeptides. A transformation protocol for yeast mitochondria is well established; nuclear genes can be recoded and introduced into the mitochondria, and mitochondrial genes can be selectively altered (Fox, Folley et al. 1991). The nuclear arginine biosynthesis gene *ARG8* has been recoded to the mitochondrial codon usage and $ARG8^{m}$ serves as a useful phenotypic reporter for mitochondrial synthesis (Steele, Butler et al. 1996). Mitochondrial genomes can be moved through crosses into different nuclear backgrounds using a process called cytoduction, which involves a mutation in the nuclear gene *KAR1* (Lancashire and Mattoon 1979). Unlike other model organisms, the complete loss of mitochondrial DNA is not fatal to yeast, as yeast can produce energy via aerobic respiration or anaerobic fermentation (Chacinska and Boguta 2000; Feldmann 2005; Fontanesi, Soto et al. 2006).

Translocation of nuclear encoded proteins

Translocation of nuclear encoded proteins into and through the mitochondrial membranes is an extensively studied and thoroughly reviewed field (Neupert and Herrmann 2007). The outer membrane contains the TOM (Translocase of mitochondrial Outer Membrane) complex, which recognizes and interacts with cytoplasmic proteins to be inserted into either the inner or outer mitochondrial membrane, or the inner membrane space. The TOM complex consists

of Tom40, Tom22, Tom5, Tom6, and Tom7, in addition to the import receptors Tom20 and Tom70 (Walther and Rapaport 2009). Tom40 is a beta-barrel protein and is thought to form a dimer pore through which many cytoplasmic translated proteins pass. A large number cytoplasmic translated proteins to be imported into the mitochondria contain a mitochondrial targeting signal as a cleavable N-terminal presequence, generally around 15-20 amino acid residues. Other proteins targeted to mitochondria contain a non cleavable internal signal for mitochondrial import (Chacinska, Koehler et al. 2009).

The inner membrane of the mitochondria contains two distinct types of TIM complexes (Translocase of mitochondrial Inner Membrane (Rassow and Pfanner 2000). Proteins are inserted into the mitochondrial inner membrane via the TIM22 complex, while the TIM23 complex functions to import proteins into the mitochondrial inner membrane and into the mitochondrial matrix (Rassow and Pfanner 2000). Proteins imported via the TIM23 complex often contain N-terminal targeting signals which are proteolytically cleaved during the import process (Vogtle, Wortelkamp et al. 2009). Import of nuclear proteins into the mitochondrial matrix first involves the TIM23 complex, then engages the ATP dependent presequence translocated-associated motor (PAM) complex. The PAM complex consists of a number of proteins – Pam16, Pam17, Pam18, Tim44, MgeI and the mtHsp70 Ssc1 (Bolender, Sickmann et al. 2008; Schiller 2009). Ssc1 is an ATPase, and provides the energy for translocation into the inner membrane.

Translation activators interact with the mitochondrial ribosome

A small number of mitochondrially encoded proteins in *S. cerevisiae* are translated by mitochondrial ribosomes, located on the matrix side of the inner mitochondrial membrane. Translation activation of the eight mitochondrial protein-coding genes has been demonstrated to function through the action of gene specific translational activators at the 5' untranslated region (UTR) (Fox 1996; Perez-Martinez, Butler et al. 2009). Seven of these protein products are subunits that make up complexes III, IV and V of the electron transport chain (Mick, Fox et al. 2011; Soto, Fontanesi et al. 2012).

Cytochrome *c* oxidase (complex IV) is the terminal electron acceptor of the electron transport chain. The process of proper cytochrome oxidase assembly is complex, involving a host of mitochondrial- and nuclear- encoded proteins (Mick, Fox et al. 2011). The core of cytochrome *c* oxidase consists of three mitochondrially encoded subunits – Cox1, Cox2 and Cox3. Translation activation of mitochondrial gene *COX1* (Cytochrome OXidase subunit 1) is dependent on *MSS51* and *PET309* (Manthey and McEwen 1995; Perez-Martinez, Broadley et al. 2003; Perez-Martinez, Butler et al. 2009), *COX2* translation activation occurs via Pet111(Poutre and Fox 1987), and *COX3* translation is activated by Pet54, Pet122 and Pet494 (Brown, Costanzo et al. 1994). The mitochondrial gene *COB,* which functions in complex III of the electron transport chain, is activated by Cbs1 and Cbs2 (Rodel 1986). The activator proteins for *COX1, COX2* and *COX3* have been shown to physically interact on the matrix side of the inner mitochondrial membrane, suggesting that translation is coordinated (Naithani, Saracco et al. 2003). The mitochondrial RNA polymerase Rpo41 interacts with primary transcripts through the protein Mft2(Rodeheffer and

Shadel 2003), which in turn has been demonstrated to physically interact with the *COX1* translation activator *PET309* (Tavares-Carreon, Camacho-Villasana et al. 2008). Mitochondrial ribosomes are bound to the inner mitochondrial membrane by the mitochondrial inner membrane insertase *OXA1,* among other interactions (Szyrach, Ott et al. 2003; Ott and Herrmann 2010). Oxa1 serves to insert mitochondrially encoded proteins into the inner mitochondrial membrane (Hell, Neupert et al. 2001). Cox1 is highly hydrophobic, containing 12 transmembrane domains (Perez-Martinez, Butler et al. 2009) and is inserted into the inner mitochondrial membrane by the translocase Oxa1 (Hell, Neupert et al. 2001).

As my thesis deals with the early steps of translational activation of *COX1*, and synthesis, stability and assembly of the Cox1 protein, via the nuclear gene *MSS51*, I will review here the current understanding in the field concerning this process.

MSS51 was initially identified as a mutant deficient in *COX1* splicing

A mutant screen by Tzagoloff et al. (1975) identified a number of nuclear genes in D273-10b yeast strain background that showed no cytochrome oxidase activity, but had functional coenzyme Q, cytochrome c reductase and ATPase (Tzagoloff, Akai et al. 1975). One of these mutants, E4-218, was characterized by Faye and Simon in 1983 (Faye and Simon 1983). The lack of respiration was correlated with a defect in splicing of the *COX1* (then referred to as oxi-3) transcript. *COX1* has 8 exons, spanning a 12883 bp genomic fragment, but has a coding sequence of only 1605 bp. When probing D273 and E4-218 mitochondrial RNA on a

Northern blot using a probe for Cox1 exon 4, Faye and Simon noticed that the E4-218 mutation caused several larger RNA products to be detected. Processing of cytochrome b pre-mRNA was unaffected by the E4-218 mutation. Faye and Simon cloned the gene responsible for the mutation via complementation, and named it *Mitochondrial Splicing System* 51, or *MSS51.* Faye and Simon concluded that *MSS51* was a protein coding gene via sequence analysis and S1 nuclease mapping.

MSS51 was identified as a translational activator of *COX1*

In 1990, Decoster et al. questioned if *MSS51* was solely involved in the splicing of *COX1* transcript (Decoster, Simon et al. 1990). They crossed a nonfunctional *mss51* allele (*mss51-1*) rho0 strain by a strain with *MSS51* and lacking mitochondrial introns, and found that respiration segregated 2:2 in the tetrads. Based on this finding, they postulated that *MSS51* was involved in translation of the *COX1* transcript. *COX1* mRNA levels in an intronless mtDNA strain with either *MSS51* or *mss51-1* were found to be unaffected via a Northern blot, but no *COX1* translation was detected with *mss51-1* via [^{35}S] methionine labeling of mitochondrial translation products over a 60 minute pulse. Decoster et al. (1990) concluded that the splicing defect of an *mss51* mutation observed by Faye and Simon was a secondary effect, as the *COX1* maturases necessary for the splicing of the *COX1* pre-mRNA are dependent on translation of *COX1.* In 2000, Siep et al. demonstrated that even with a short 5 minute pulse, no *COX1* translation can be detected in *mss51Δ* in a [^{35}S] methionine labeling of mitochondrial translation products (Siep, van Oosterum et al. 2000). *MSS51* has been demonstrated to be a peripheral inner membrane

protein (Siep, van Oosterum et al. 2000; Barrientos, Zambrano et al. 2004). Perez-Martinez et al. demonstrated that *MSS51's* translational activation occurs through the *COX1* 5' UTR, and that Mss51 is present at or near rate limiting levels in the cell (Perez-Martinez, Butler et al. 2009).

MSS51 has an additional role in the synthesis of Cox1p

As previously discussed, translational activation of *COX1* occurs via the *COX1* 5' UTR by the nuclear encoded factors *MSS51* and *PET309*. However, *MSS51* is distinct among other *S. cerevisiae* mitochondrial translational activators in that it has an additional function, in its physical interaction with newly synthesized Cox1p, likely through the C-terminal residues (Perez-Martinez, Broadley et al. 2003; Shingu-Vazquez, Camacho-Villasana et al. 2010). Additionally, *MSS51* has no currently defined domains or motifs that are responsible for its functions.

In 2003, Perez-Martinez et al. used the reporter gene $ARG8^m$ to ascertain the function of *MSS51* (Perez-Martinez, Broadley et al. 2003). $ARG8^m$ was fused to the C terminus of full length *COX1*, creating *COX1(1-512)::*$ARG8^m$ mtDNA, and also inserted in the place of *COX1*, creating *cox1Δ::*$ARG8^m$ mtDNA. Using these mtDNA constructs, growth on CSM-ARG plates could serve as a translational readout of *COX1* expression. Perez-Martinez found that when either *MSS51* or *PET309* was deleted, a strain bearing *COX1(1-512)::*$ARG8^m$ mtDNA was both unable to respire and unable to grow on CSM-ARG. Interestingly, when an anti-Arg8 western blot was performed with *MSS51* in a strain bearing *COX1(1-512)::*$ARG8^m$ mtDNA, two bands were detected, a large band presumably corresponding to Cox1-$Arg8^m$ fusion protein, and

a smaller band corresponding to Arg8^{m}. There is a processing site near the N terminus of Arg8^{m} which causes it to be cleaved from the Cox1 message.

Perez-Martinez also created a mtDNA arrangement critical to the mutant screen discussed in Chapter 2, in which *COX1* is flanked by the 73 and 119 bp of the *COX2* 5' and 3' untranslated leaders, and placed 295 bp upstream of the endogenous *COX2* locus in the opposite orientation, and *ARG8^{m}* has replaced *COX1* at the *COX1* locus. In a strain bearing this *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, *COX1* is under translation activation control from the *COX2* translational activator *PET111*. However, in an *mss51Δ*, a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA was still unable to synthesize *COX1*. Therefore, *MSS51* has a role in *COX1* synthesis and/or assembly, in addition to its role at translation activation at the *COX1* 5' UTR. In contrast, a strain containing a *pet309Δ* was unable to grow on CSM-Arg, as translation activation of *cox1Δ::ARG8^{m}* is controlled by *COX1* translational activators *PET309* and *MSS51.* However, a *pet309Δ* strain with this rearranged mtDNA background was able to respire, indicating that in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, synthesis and assembly of Cox1 is independent of translation activation at *COX1.*

Perez-Martinez showed that Mss51 physically interacted with newly synthesized, full length Cox1p (Perez-Martinez, Broadley et al. 2003). Also included in this complex is the nuclear gene product Cox14 (Barrientos, Zambrano et al. 2004). Cox14 was demonstrated to physically interact with newly synthesized Cox1 and with Mss51, and interaction between Mss51 and Cox14 is dependent on Cox1

(Perez-Martinez, Butler et al. 2009). Fontanesi et al. reported that Mss51 was found in a 120 kDa complex with the mtHsp70 chaperone Ssc1, which occurs independently of Mss51's association with Cox1 (Fontanesi, Soto et al. 2009). See Figure 1.1 for a model of Mss51 function.

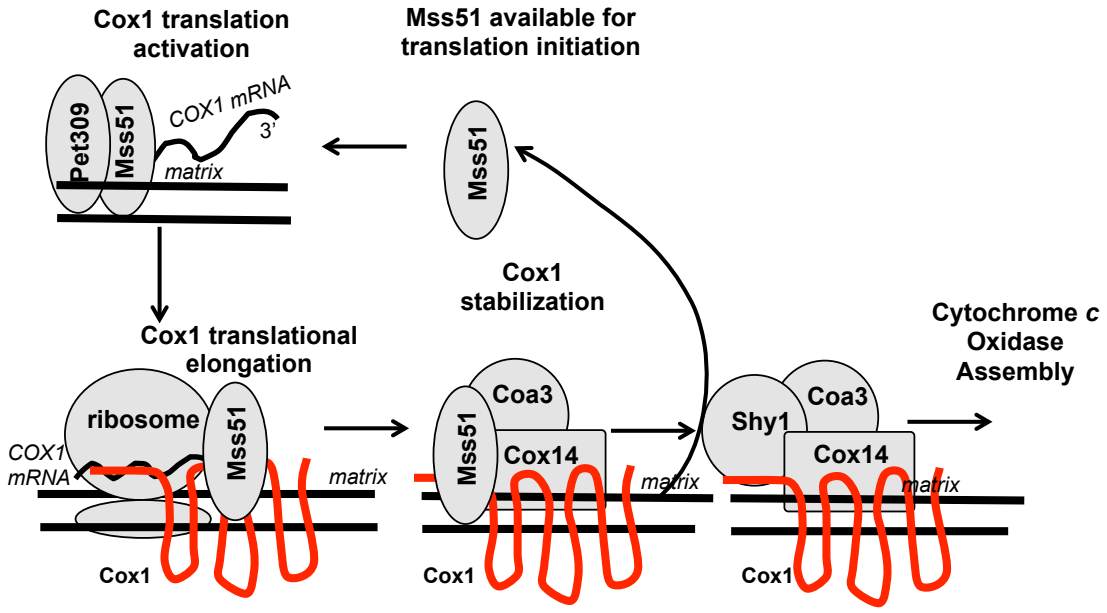

Figure 1.1 – Model for Mss51 function. Mss51 and Pet309 activate translation at the *COX1* 5' UTR. Mss51 binds to nascent Cox1 along with assembly factors Cox14 and Coa3. Mss51 has a role in assembly of Cox1 into cytochrome *c* oxidase. Mss51 releases from the intermediate assembly complex to activate subsequent rounds of translation at the *COX1* 5' UTR. Cox1 assembly cofactors Shy1 and Cox14 remain associated with Cox1 as it is assembled into larger complexes (Mick, Wagner et al. 2007). Figure modified from Perez-Martinez *et al*. 2009 (Perez-Martinez, Butler et al. 2009).

COX1 expression is under negative feedback regulation, involving *COX14* and *COA3*

COX1 is the largest subunit of cytochrome *c* oxidase (Gray, Lang et al. 2004). Proper assembly of Cox1 into cytochrome *c* oxidase is critical both to produce a functioning respiratory complex, and to protect the cell from oxidative damage (Khalimonchuk, Bestwick et al. 2009). Khalimonchuk found that deleting *COX2* or *COX3* rendered the cells sensitive to 6 mM H_2O_2, but *cox1Δ* abrogated this sensitivity.

Barrientos et al. observed that when downstream assembly factors of Cox1 are deleted, translation of *COX1* is significantly downregulated. Deletion of *COX10* or *COX15*, involved in heme a biosynthesis, caused a dramatic decrease in *COX1* translation, even though these enzymes are not directly involved in *COX1* translation activation (Barrientos, Zambrano et al. 2004). As unassembled Cox1 has been shown to be a pro-oxidant, downregulation of Cox1 when assembly of cytochrome c oxidase is blocked makes logical sense (Khalimonchuk, Bird et al. 2007). Interestingly, when the Cox1 assembly factor *COX14* is deleted, the strain is unable to respire but translation of *COX1* is not affected.

COA3 also functions in *COX1* feedback regulation. *COA3* is a nuclear encoded gene that functions in the mitochondria, and was identified in a pull down of Shy1 (Mick, Vukotic et al. 2010). Mick et al. determined that Coa3 and Cox14 were resistant to carbonate extraction and therefore were integral membrane proteins (Mick, Vukotic et al. 2010). A *coa3Δ* was unable to respire, but when the

mitochondrial translation products were labeled, *COX1* translation was not significantly decreased.

Shy1 is a Cox1 assembly factor and is involved in heme a3 insertion

SHY1 is a mitochondrial protein involved in *COX1* biogenesis. Shy1 interacts relatively early in the Cox1 assembly pathway, as it was found in a complex containing Mss51 and Cox14 (Mick, Wagner et al. 2007). While *MSS51* disengages from the Cox1 assembly complex, Shy1 remains bound when fully assembled Cox forms 'supercomplexes' with complex III of the electron transport chain. *SHY1* is the yeast homolog of the human gene *SURF1*(Barrientos, Korr et al. 2002). *SURF1* mutations have been demonstrated to be responsible for Leigh's syndrome, a rare mitochondrial disorder (Rossi, Biancheri et al. 2003). In W303 and S288C *S. cerevisiae* strain backgrounds, *shy1Δ* mutants fail to respire normally, but a *shy1Δ* does not display a respiratory defect in the D273-10B background, for reasons that are currently unknown. Barrientos et al. found that a *shy1Δ* in W303 background can be suppressed by *mss51* alleles *mss51-F199I* and *mss51-T167R* on a single copy vector, or *MSS51* when present in two or more copies(Barrientos, Korr et al. 2002). Despite this suppression, Barrientos et al. were unable to detect a direct interaction between Mss51p and Shy1 via sucrose gradient sedimentation. However, in 2007 Mick et al. detected Shy1 pulled down with a TAP tag on *MSS51*, so there is evidence that Mss51 and Shy1 are present in the same complex (Mick, Wagner et al. 2007).

Mss51 is sequestered in Cox1 intermediate assembly complexes

Mss51 has been found in cytochrome *c* oxidase intermediate assembly complexes with Cox14, Shy1, Coa3 and Coa1 (Barrientos, Zambrano et al. 2004; Mick, Wagner et al. 2007; Pierrel, Bestwick et al. 2007; Mick, Vukotic et al. 2010). *COA3* behaves similar to *COX14* with regards to the relatively high levels of *COX1* translation in a *coa3Δ,* despite the inability of a *coa3Δ* to assembly cytochrome oxidase. It has been proposed that Cox14 and Coa3 physically interact with Mss51 to keep Mss51 in the sequestered state with Cox1, and therefore unable to activate new rounds of *COX1* translation (Perez-Martinez, Broadley et al. 2003; Barrientos, Zambrano et al. 2004; Mick, Vukotic et al. 2010). Mick et al. proposed a multistep process for downregulation of *COX1* translation (Mick, Fox et al. 2011). In their model, Mss51, Cox1, Coa3 and Cox14 are initially present in a 250 kDa complex which may loosely sequester Mss51, but Mss51 is not completely inactivated via sequestration. Coa1 then interacts with this complex and is thought to serve to assist further sequestering Mss51 (Figure 1.2).

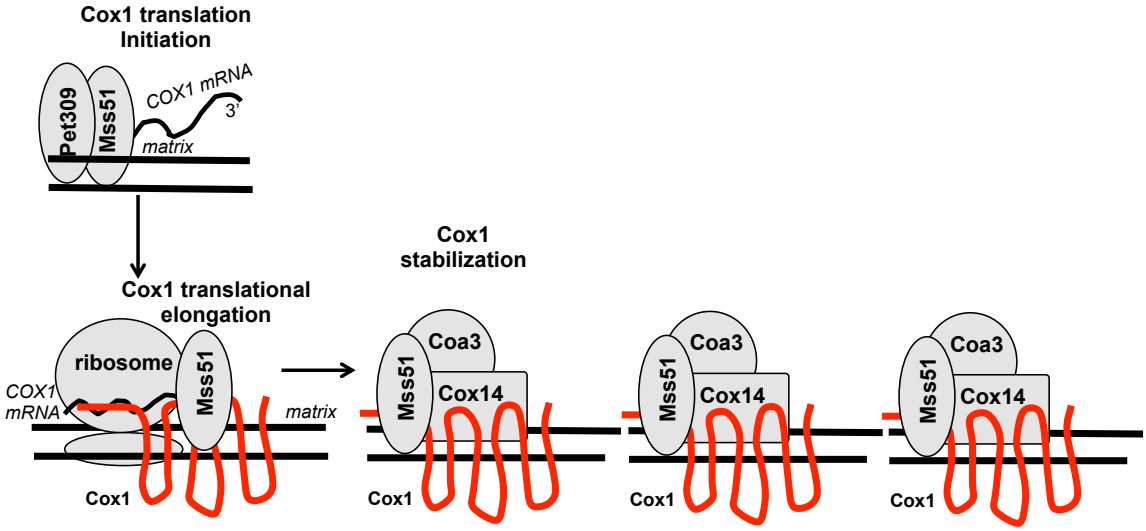

Figure 1.2 – Physical sequestration of Mss51 in Cox1 intermediate assembly complexes. When cytochome *c* oxidase assembly is blocked downstream of Cox1, Mss51 has been proposed to accumulate in intermediate assembly complexes, rendering it unable to activate new rounds of translation at the *COX1* 5' UTR.

Coa1 plays an additional role in the recruitment of Shy1. Pierrel et al. found that in a *shy1Δ* strain, *COX1* translation is significantly decreased (Barrientos, Korr et al. 2002; Pierrel, Bestwick et al. 2007). *COX1* translation was significantly increased (although not to WT levels) in a strain bearing both *shy1Δ* and *coa1Δ* (Pierrel, Bestwick et al. 2007). It has therefore been proposed that the absence of Shy1 caused Mss51 to be largely sequestered, but that the sequestration was relaxed when Coa1 was also deleted (Pierrel, Bestwick et al. 2007). However, when Shy1 is present, it may act as a factor that causes Mss51 to dissociate. Other studies suggest nuclear assembly intermediates Cox5 or Cox6 might be involved in the release of Mss51 (Barrientos, Zambrano et al. 2004; Shingu-Vazquez, Camacho-Villasana et al. 2010; Mick, Fox et al. 2011).

Cox5 and Cox6 are thought to be the first nuclear subunits of cytochrome *c* oxidase to associate with Cox1, and are thought to play a role in stabilizing Cox1 (Horan, Bourges et al. 2005; Fontanesi, Jin et al. 2008) (Figure 1.3). It was found that in *S. cerevisiae,* a *cox6Δ* causes a dramatic reduction in *COX1* translation. Shingu-Vasquez proposed that the addition of Cox5 or Cox6, or the insertion of the heme groups to Cox1, could cause a conformational change in the Cox1 complex, thus promoting the dissociation of Mss51 (Shingu-Vazquez, Camacho-Villasana et al. 2010).

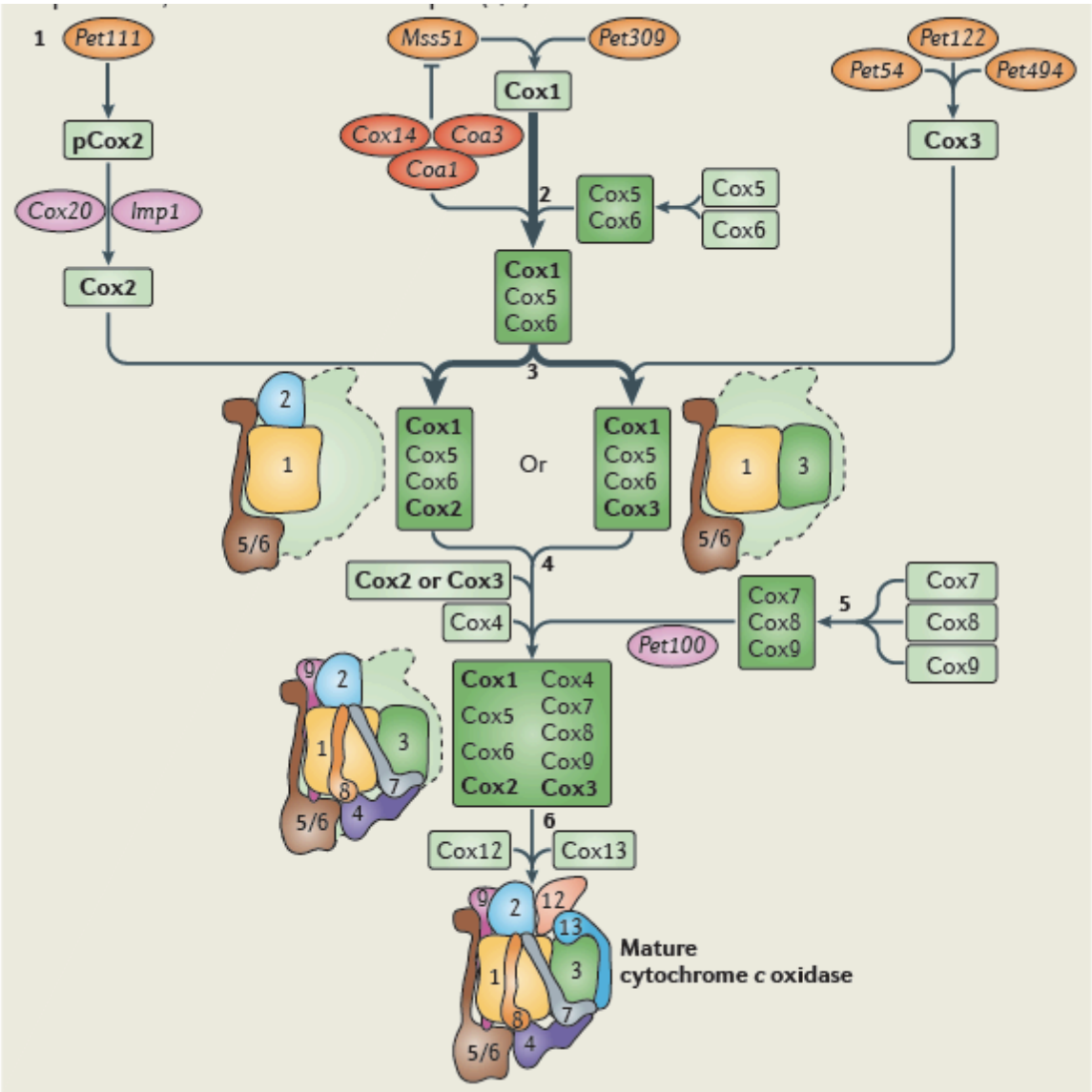

Figure 1.3 - Overview of cytochrome *c* oxidase assembly. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology. Mick, D. U., T. D. Fox, et al. (2011). "Inventory Control: cytochrome c oxidase assembly regulates mitochondrial translation." Nature reviews. Molecular cell biology **12**(1): 14-20. Copyright 2011.

Hemylation of Cox1 is Carried out by Coa2

Coa2 is a small nuclear encoded mitochondrial protein, identified as a multicopy suppressor of the respiratory defect caused by a *coa1Δ* in the W303 strain background (Pierrel, Khalimonchuk et al. 2008). A *coa2Δ* was found to be unable to support respiration, and the defect was specifically due to a cytochrome *c* oxidase deficiency. In a *coa2Δ* cell, Cox1p is highly unstable and the cells are sensitive to H2O2, which suggests an exposed heme group in Cox1. The H2O2 sensitivity of a *coa2Δ* was abrogated by a *shy1Δ*, which suggests that *COA2* and *SHY1* function at or near a similar step. The respiratory defect of a *coa2Δ* was suppressed by a gain of function N196K mutation in Cox10, a farnesyl transferase, or by disruption of the Oma1 metallopeptidase (Bestwick, Khalimonchuk et al. 2009). Cox10, along with Cox15, are involved in heme *a* biogenesis (Glerum and Tzagoloff 1994; Wang, Wang et al. 2009).

Cox1 Degradation by Oma1

Khalimonchuk et al. proposed that the Oma1 metalloproteinase is a quality control enzyme of cytochrome *c* oxidase assembly (Khalimonchuk, Jeong et al. 2012). The degradation of Cox1 by Oma1 was found to occur only in a *coa2Δ* strain; in a *COA2* strain Oma1 did not degrade Cox1. Khalimonchuk suggested that in the absence of *COA2,* Oma1 is activated and the structure of Cox1 is altered to an extent that allows for specific proteolysis of Cox1 by Oma1. This is the strongest data presented thus far that suggests a mechanism for Cox1 proteolysis.

Mss51p release from intermediate assembly complexes

I feel that the model for sequestration of Mss51 is intriguing, but the precise order of events concerning the release of Mss51 from the Cox1 assembly intermediates is not well described at the present time. It is difficult to precisely define what proteins are together in the complex. As there is no *in vitro* translation system for yeast mitochondria, this system cannot be studied using purified components. An undergraduate in the Fox lab, Whitney Thompson, is currently working on temperature sensitive *mss51* mutants in wild type mtDNA that would be unable to respire at restrictive temperatures, hopefully stalling the Cox1 assembly complex. Whitney has identified several such mutants, one of which has relatively high levels of *COX1* translation and steady state Cox1p. It would prove quite interesting to examine what proteins are present in the stalled Cox1 assembly complex, if it can be detected.

In summary

As *MSS51* has no currently defined domains or motifs, it is of relevant biological interest to determine which parts of the gene are responsible for its functions in translation activation and Cox1 assembly. The *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA constructed by Perez-Martinez provides an excellent tool with which to perform a mutant screen to find *mss51* alleles that separate the functions of *MSS51. cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA provides separate and distinct phenotypes for the translational activation at the *COX1* 5' UTR and Cox1p synthesis into cytochrome *c* oxidase.

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Chapter 2

A screen for separation-of-function *mss51* mutants in a strain bearing a *cox1Δ::ARG8^m cox2Δ::COX1* mitochondrial genome

INTRODUCTION

The goal of this mutagenesis screen was to find *mss51* mutant alleles that separate the function of translation activation at the *COX1* 5' UTR from the function of interacting with and assembling the nascent Cox1p. By studying these *mss51* mutants I hope to elucidate domains, motifs or residues of *MSS51* that are involved in its functions.

To address this question, a rearranged *cox1Δ::ARG8^m cox2Δ::COX1* mitochondrial genome was utilized. This mitochondrial rearrangement was generated by Xochitl Perez-Martinez, a former post-doctoral researcher in the Fox lab (Perez-Martinez, Broadley et al. 2003). Perez-Martinez created a mitochondrial genome that contained *ARG8^m* coding sequence flanked by the *COX1* UTR's, at the *COX1* locus. *COX1* was placed 295 bp upstream of the *COX2* locus but in the opposite orientation, and flanked by 73 and 119 bp of the *COX2* 5' and 3' UTRs, respectively In a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, *MSS51* is able to support growth on medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG). An *mss51Δ* was both unable to grow on CSM-ARG and unable to

support respiration, while a *pet309Δ* was Arg- and was able to support respiration. Deletion of the *COX2* translational activator *PET111* abolished both *COX1* and *COX2* synthesis, but allowed for growth on CSM-Arg. This demonstrates that in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, *ARG8^{m}* translation is dependent on the activation activity of *MSS51* and *PET309* at the *COX1* 5' UTR, while translation activation of *COX1* through the *COX2* 5' UTR under translation activation control of *PET111.* [^{35}S] methionine labeling of mitochondrial translation products in strains bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA supported the observed growth phenotypes - an *mss51Δ* showed no *ARG8^{m}* or *COX1* translation, a *pet309Δ* did not affect *COX1* translation but abrogated *ARG8^{m}* translation, and a *pet111Δ* did not affect *ARG8^{m}* translation, but eliminated *COX1* translation. Therefore, *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA is capable of providing distinct phenotypes that report on translation activation at the *COX1* 5' UTR and synthesis and assembly of Cox1p.

In a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, an *mss51* mutant that is capable of interacting with the *COX1* 5' UTR but not assembling Cox1 would be prototrophic for arginine biosynthesis, but would be unable to support respiration. Alternatively, an *mss51* mutant that fails to interact with the *COX1* 5' leader but could assemble Cox1p would be unable to grow on CSM-ARG but would be capable of supporting respiratory growth.

A second mtDNA arrangement used in this project is *COX1(1-512)::ARG8^{m}* mtDNA. In this arrangement, the *ARG8^{m}* reporter is translationally fused to the C terminus of full length *COX1*, at the *COX1* locus. This mitochondrial construct

provides a phenotypic reporter for *COX1* translation. *ARG8^{m}* contains a mitochondrial processing site for the pre-Arg8p matrix targeting signal. In a strain bearing *COX1(1-512)::ARG8m* mtDNA, both *PET309* and *MSS51* are necessary for biosynthesis of arginine and the ability to support respiration (Perez-Martinez, Broadley et al. 2003).

METHODS AND MATERIALS

Strains, media and genetic methods

Saccharomyces cerevisiae strains used in this chapter are listed in Table 2.4. Standard genetic methods and media recipes were as previously described (Fox, Folley et al. 1991). Complete fermentable medium was YPAD (containing 2% glucose, 20.27 mg adenine / L). Non-fermentable medium was YPAEG (3% glycerol, 3% ethanol, 20.27 mg adenine / L). Minimal medium was synthetic complete (0.67% yeast nitrogen base, 2% glucose) lacking adenine.

PCR mutagenesis

Mutagenic PCR conditions are shown in Table 2.1. Primer AS1 sequence is 5' TGC AAG GCG ATT AAG TT 3' and T3 is 5' ATT AAC CCT CAC TAA AG 3'. Plasmid pCB16 is pRS316 backbone (Sikorski and Hieter 1989) with *MSS51::3xHA* cloned into MCS using restriction enzymes HindIII and XbaI (Invitrogen). It is important to note that every mutant *mss51* allele utilized throughout this thesis contains a triple HA epitope tag on the C terminus, except those that truncated the protein.

Transformation of Yeast Strains

Transformation of yeast strains was performed according to the Frozen-EZ Yeast Transformation II Kit (ZYMO Research). Plasmid vector pRS316 was linearized using HindIII and XbaI restriction enzymes (Invitrogen) and quantitated visually on 1% agarose gels. 200 ng linearized vector and 800 ng mutagenized PCR product was transformed into strain XPM174 to integrate mutagenized *mss51* into the vector by

recombination (Muhlrad, Hunter et al. 1992). Mutant *mss51* alleles were integrated at the *MSS51* chromosomal locus by digesting *mss51* from the vector backbone with HindIII and XbaI restriction enzymes (Invitrogen), and transforming the *mss51* fragment into strain CAB305 (*mss51Δ::LEU2 COX14::3xMYC,* wild type mtDNA) and screening for transformants that became Leu (-) by plating transformants to YPAD medium and replica plating to medium lacking leucine. Leu (-) transformants were patched to YPAD medium and printed to medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) to assess their phenotypes. Genomic DNA was prepared from putative integrated strains and PCR was performed on *mss51* using primers MSS51F4 5' CCC AAA GCT TCG TCT TGG CTG GAA CTC AAC CG 3' and MSS51R5 5' CCA AAA GCT TCC TAA TAT AGT CCG AAA AGG AAA GG 3' to verify that integration had occurred at the correct chromosomal location. Additionally, the *mss51* PCR products were sequenced using primers SAB-8F 5' GGA GTC TGA CAA GAG AC 3' and Mss51F2 5' GTT GGA GAA TAG ATC TTT ACC AAC C 3' to verify the correct *mss51* allele was integrated.

Screening protocol

Serial dilutions of XPM174 transformed with linearized vector and mutagenized *mss51* PCR product were plated onto CSM-Ura plates to a desired density of ~750 transformants per plate. CSM-Ura plates were replica plated onto CSM-Arg and YPAEG plates. To account for phenotypic lag, plates were printed twice to CSM-Arg, denoted in Figure 2.1 as CSM-Arg 2x. After printing to CSM-Arg 2x and YPAEG, CSM-

Arg 2x plates were printed to YPAEG plates, and YPAEG plates were printed to CSM-Arg 2x plates.

Sequencing *mss51*

mss51 plasmids were isolated from yeast using the Yeast Plasmid Miniprep protocol (Rose 1986). Plasmids were transformed into DH5alpha cells (Invitrogen) according to the Invitrogen protocol and plated to LB+Amp agar plates. Transformants were grown in 5 mL liquid LB+Amp and E. coli minipreps were performed using the QIAprep Spin Miniprep Kit (QIAGEN). Plasmids were sequenced at the Cornell Life Sciences Core Laboratories Center using primers SAB-8F 5' GGA GTC TGA CAA GAG AC 3' and Mss51F2 5' GTT GGA GAA TAG ATC TTT ACC AAC C 3'.

Generation of rho0 strains

Strains were made rho0 via 3 days growth in 5 mL CSM + 25 ug/mL EtBr at 30ºC. The EtBr cells were diluted 1:100 in the same media and incubated an additional 3 days. 2x EtBr treated cells were streaked to YPAD plates for single colonies, printed to YPAEG media to test for respiratory deficiency. Additionally, the single colonies were mated to a verified rho0 tester strain and the diploid printed to YPAEG to verify that the lack of respiration in the haploid was caused by the loss of mtDNA.

RESULTS AND DISCUSSION

Initial screen mutant screen

Table 2.1 – PCR conditions for error-prone PCR mutagenesis of *MSS51*

	Volume (uL)
Buffer, no MgCl2 (10X)	5
Template (10 ng/uL)	1
Primer 1 - AS1 (12.5 um/uL)	1
Primer 2 - T3 (12.5 um/uL)	1
dATP (10 mM)	1
dCTP (100 mM)	1
dTTP (100 mM)	1
dGTP (100 mM)	1
MgCl2 (50 mM)	3
MnCl2 (100 mM)	1.75
Invitrogen Taq	0.5
dH2O	QNS 50 uL

Mutagenic PCR of *MSS51* was performed in order to randomly generate *mss51* mutants using plasmid pBC16 as a template with primers AS1 and T3. (Muhlrad, Hunter et al. 1992). Transformants were generated in strain XPM174 (*mss51Δ::LEU2, cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA) that expressed the mutated *mss51* gene from the pRS316 vector backbone. A screen was performed for colonies that were able to grow on CSM-ARG but not YPAEG, and alternatively colonies that were able to grow on YPAEG but not CSM-Arg (Figure 2.1).

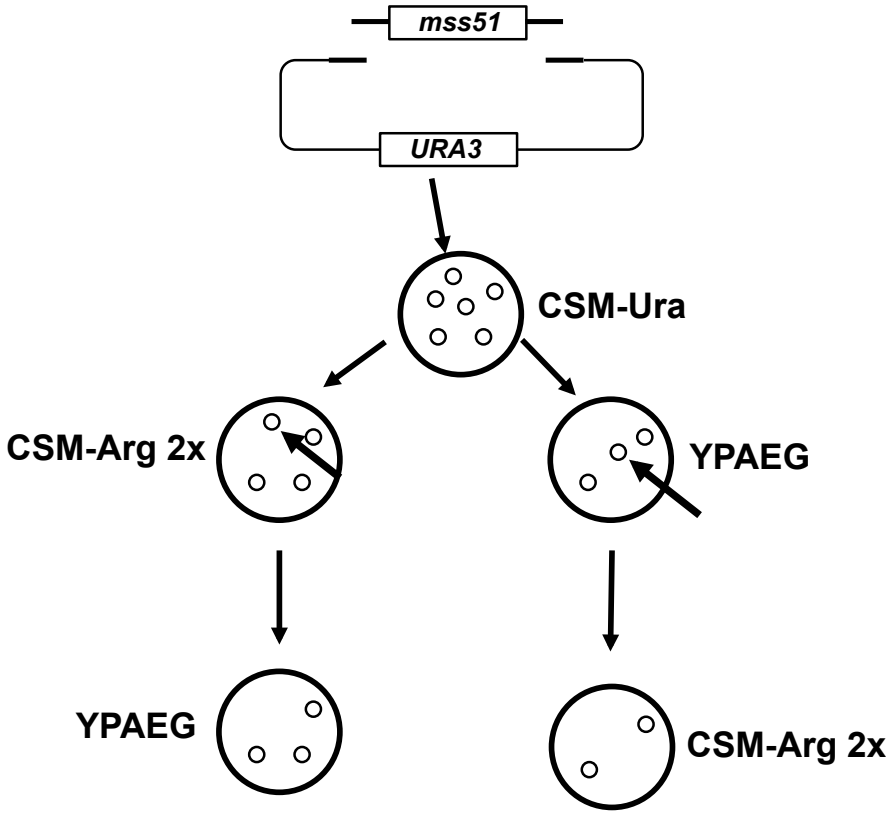

Figure 2.1 – A screen for separation of function mutants in *MSS51.* The mutant screen utilized a rearranged *cox1Δ::ARG8^{m} cox2Δ::COX1* mitochondrial genome. Transformants containing mutagenized *mss51*were selected on medium lacking uracil (CSM-Ura). Transformants were replica plated in parallel to medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG). Transformants were replica plated to CSM-Arg twice due to phenotypic lag, represented as CSM-Arg 2x throughout the thesis. Transformants were then replica plated to the converse medium, as indicated. After each printing, plates were incubated for 2 days at 30ºC. Desired mutants were colonies that were able to grow on CSM-Arg but were unable to respire, or colonies that were able to respire but unable to grow on CSM-Arg (indicated by arrows).

When a potentially interesting candidate colony was identified, it was isolation streaked from the initial CSM-Ura colony onto CSM-Ura and reprinted to CSM-Arg and YPAEG to verify the phenotype. If the initially observed phenotype persisted, the plasmid was isolated from the yeast cells, transformed into E. coli, and retransformed into strain XPM174 to verify if the initially observed phenotype persisted when the plasmid was reintroduced. It was found that of the initial 82 Arg+ Pet- mutants identified, 13 did not recapitulate the mutant phenotype when retransformed into XPM174. 2 of these plasmids conferred an Arg- and Pet- phenotype when retransformed, so the initial Arg+ Pet- phenotype was likely due to an Arg+ mutation in a nuclear gene other than *mss51,* and *mss51* was nonfunctional on the plasmid. The other 11 plasmids conferred an Arg+ and Pet+ phenotype when retransformed, so the initial Arg+ Pet- phenotype was likely due to a Pet- mutation in a nuclear gene, and *MSS51* on the plasmid was fully functional. 13 plasmids containing *mss51* alleles which when retransformed into XPM174 conferred an Arg+ Pet- phenotype are frozen as *E. coli* stocks, named pZV30 – pZV42. *mss51* from these plasmids has not been sequenced, nor have these plasmids been transformed into XPM76 or XPM89 to assess their phenotype is strains bearing *COX1(1-512)::ARG8*^{m} or wild type mtDNA. Five of the *mss51* plasmids were transformed into strain XPM76 (*mss51Δ::LEU2, COX1(1-512):: ARG8*^{m} mtDNA) and also into XPM89 (*mss51Δ::LEU2,* wild type mtDNA).

Table 2.2 – Summary of statistics from mutant screen

Approximate number of URA+ colonies examined	6x10^5
Approximate percent of Arg- Pet- colonies	8%
Approximate percentage of Arg+ Pet- colonies	0.014%
Number of Arg- Pet+ colonies	0
Approximate percentage of false Arg+ Pet- positives	16%

A summary of the mutant screen statistics is found in Table 2.2. I estimate that approximately 600,000 initial transformants on CSM-Ura were selected. The mutant screen was carried out in several iterations. I found that approximately 8% of the colonies were Arg- and Pet-, which could be caused by either a nonfunctional *mss51* mutant, by the host strain XPM174 going rho- due to the transformation procedure, or failure to integrate *mss51* into the vector after transformation. The overwhelming majority of the cells screened were Arg+ and Pet+; I estimate that approximately 0.014% colonies were Arg+ and Pet- in a strain bearing , *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA. However, the standards for selecting mutant colonies was quite strict; if a colony was Arg+ and weakly Pet+, or if there was uncertainty concerning whether a mutant phenotype was present, the colony was ignored. No *mss51* mutants were identified that were Arg- and Pet+, despite careful

and thorough efforts to identify such a mutant. A potential reason why this class of mutants was not identified will be discussed in Chapter 5.

One class of *mss51* mutants was identified that strongly respired when the *COX1* 5' UTR was present in *cis* to the *COX1* coding sequence.

Initial efforts focused on five plasmid borne *mss51* mutants – *mss51-51, -52, -101, -102,* and *-103* (Table 2.3, Figure 2.2). Plasmid pZV2 contains allele *mss51-51,* plasmid pOAB2 contains allele *mss51-52,* plasmid mss51-101p contains allele *mss51-101,* plasmid mss51-102p contains allele *mss51-102*, and plasmid mss51-103p contains allele *mss51-103.* These five mutants were initially chosen to pursue only because they were among the first identified. When on a plasmid, three of these mutants, *mss51-101, -102,* and *-103* were Arg+ and Pet- in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, Arg+ and Pet+ in a strain bearing *COX1(1-512)::ARG8^m* mtDNA, and Pet+ in wild type mtDNA after 3 days incubation at 30ºC (Figure 2.3). The fact that *mss51-101, -102* and *-103* were able to respire strongly when the *COX1* 5' UTR was present in *cis* to the *COX1* coding sequence - as in strains bearing *COX1(1-512)::ARG8^m* and wild type mtDNA - was surprising, and will be pursued in Chapter 5.

Table 2.3 - *mss51* alleles identified in the mutant screen

mss51-51		*mss51-52*		*mss51-102*		*mss51-103*	
bp	aa	bp	aa	bp	aa	bp	aa
A485G	D162G	T44C	F15S	A883G	T295A	T33C	silent
A497G	Y166C	T190C	W64R	A1122G	silent	C152A	S51Y
A523G	T175A	A194G	D65G	G1280A	G427D	T291C	silent
T570C	silent	A736G	I246W			T649C	S217P
T678C	silent	C789T	silent			A1191G	silent
C796	frameshift	T868C	Y290H				
		A908T	E303V				
		A1146G	silent				

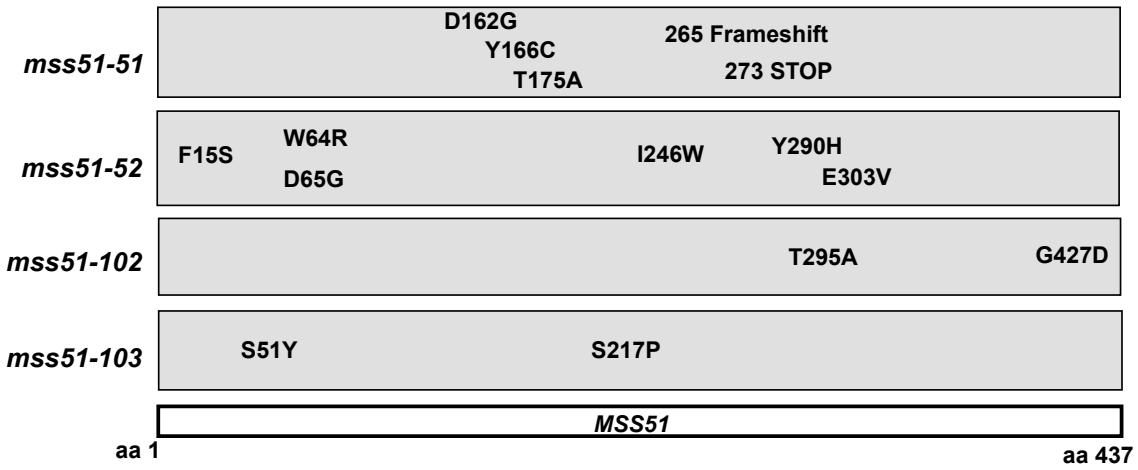

Figure 2.2 – Amino acid substitutions in four *mss51* alleles.

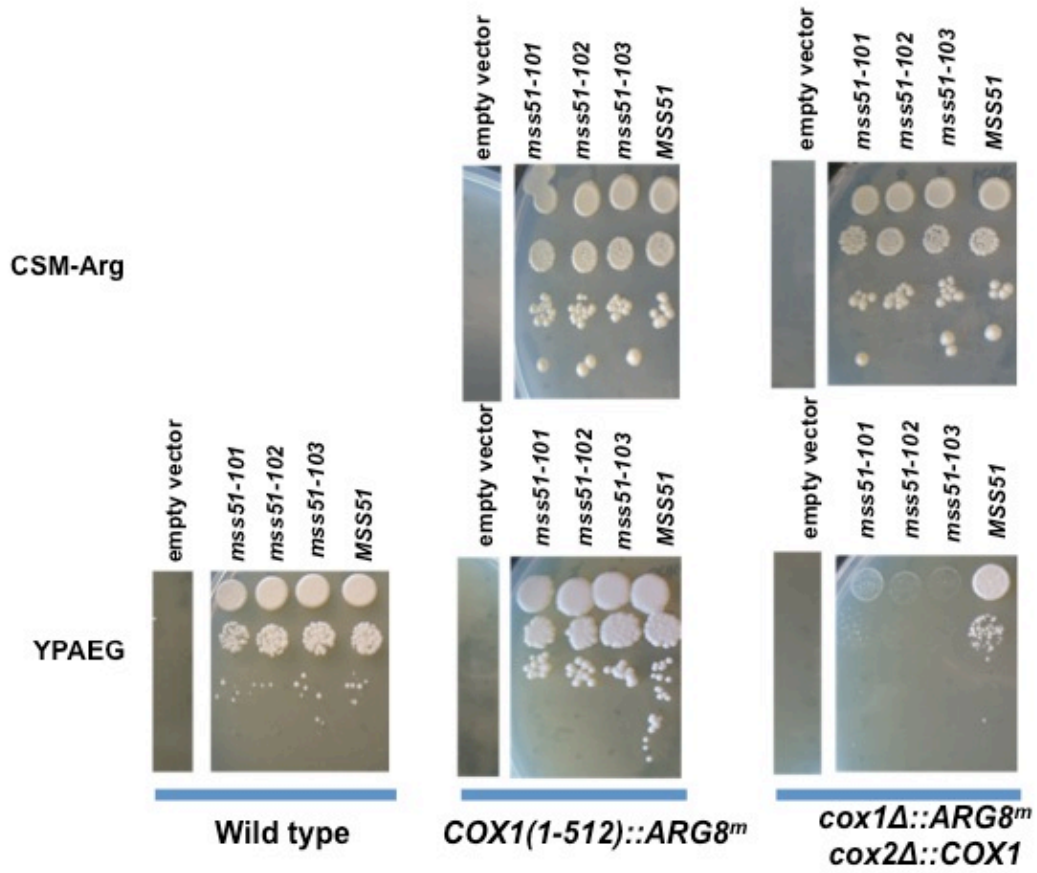

Figure 2.3 – Plasmid borne *mss51-101, mss51-102* and *mss51-103* weak support respiration in strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA and containing *mss51* alleles on a ARS CEN vector were spotted medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. Strains bearing wild type mtDNA were: empty vector, XPM89 + pRS316; *mss51-101*, XPM89 + mss51-101p; *mss51-102*, XPM89 + mss51-102p; *mss51-103,* XPM89 + mss51-103p*; MSS51,* XPM89 + pCB16. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: empty vector, XPM76 + pRS316; *mss51-101*, XPM76 + mss51-101p; *mss51-102*, XPM76 + mss51-102p; *mss51-103,* XPM76 + mss51-103p*; MSS51,* XPM76 + pCB16. Strains bearing *cox1Δ::ARG8*^{m}

cox2Δ::COX1 mtDNA were: empty vector, XPM174 + pRS316; *mss51-101*, XPM174 + mss51-101p; *mss51-102*, XPM174 + mss51-102p; *mss51-103,* XPM174 + mss51-103p*; MSS51,* XPM174 + pCB16. This dilution series has not been repeated. See Table 2.4 for complete genotypes.

Integrants were identified among Leu(-) transfomrants by their growth phenotypes on CSM-Arg and YPAEG (Methods and Materials), then sequenced to ensure that *mss51* integrated at the correct chromosomal locus. To ascertain the mutant phenotypes in *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA when integrated, all mutants were made rho0 and crossed to strain XPM171 (*MSS51, cox1Δ::ARG8^m cox2Δ::COX1* mtDNA). Diploids were selected, sporulated and tetrads dissected. To ascertain the mutant phenotype of strains bearing *COX1(1-512)::ARG8^m* mtDNA when the *mss51* alleles were integrated, all rho0 integrants were also crossed to XPM78 (*MSS51*, *COX1(1-512)::ARG8^m* mtDNA). Diploids were selected, sporulated and tetrads dissected. When the integrants were crossed into these mtDNA backgrounds, *mss51* was sequenced from tetrads that had the expected mutant phenotype to ensure that the proper *mss51* mutant was present. Additionally, total protein was prepared from each strain, and anti-HA and anti-MYC ECL Western blots were performed to verify the presence of mutant *mss51-HA* and *COX14::MYC.*

It was found that when integrated, *mss51-101* in a strain with *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA background showed a weak Arg+ and weak Pet+ phenotype after 3 days growth (Figure 2.4). As this is no longer the desired mutant phenotype, *-101* was disregarded. The most apparent explanation of why *-101* failed to respire

on a plasmid but was able to respire when integrated is an alteration translation rate of *mss51-102*. As Mss51p is known to be at or near rate limiting levels, I would assume that levels of Mss51-102p is increased when integrated, which leads to a weak Pet+ phenotype after 3 days (Perez-Martinez, Butler et al. 2009). This hypothesis could be tested via quantitative fluorescence Western blot analysis, but I have not done so.

mss51-102 and *-103* showed an Arg+, Pet- phenotype after 3 days when crossed into a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1*mtDNA (Figure 2.4), which indicated that these mutant are worthy of further study. The Arg+ phenotype of *mss51-102* and *-103* in this mtDNA background demonstrates that *mss51* is capable of activating translation at the *COX1* 5' UTR. After an extended 8 day incubation, it was found that *mss51-102* and *-103* were able weakly respire, so respiration is not completely deficient when the *COX1* 5' UTR is present in *trans* to the coding sequence, just significantly diminished.

When crossed into a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA, *mss51-102* and *-103* are strongly Arg+ and Pet+, which indicates that the mutant *mss51* is capable of activating translation at the *COX1* 5' UTR, fully reading through the *COX1* coding sequence, and properly assembling Cox1 into cytochrome *c* oxidase. Similarly, these mutants are Pet+ in a strain when integrated in a strain bearing wild type mtDNA, which matches the original plasmid phenotype observed.

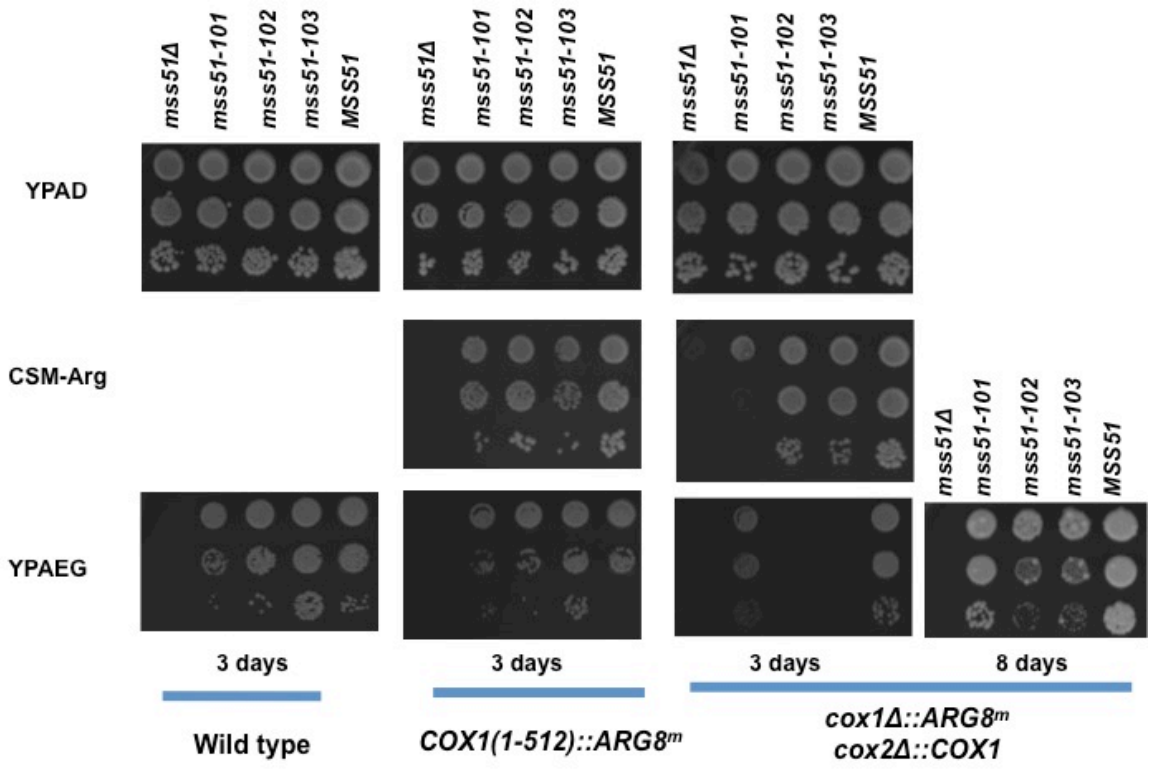

Figure 2.4 – Integrated *mss51-102* and *mss51-103* weakly support respiration in strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 or 8 days at 30ºC as indicated. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-101*, CAB323; *mss51-102*, CAB324; *mss51-103,* CAB325*; MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM76; *mss51-101*, ZV14; *mss51-102*, ZV18; *mss51-103,* ZV25*; MSS51,* XPM78. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-101*, ZV26; *mss51-102*, ZV27; *mss51-103,* ZV22*; MSS51,* XPM171. This dilution series has been repeated twice. See Table 2.4 for complete genotypes.

One class of *mss51* mutants was identified that failed to support respiration in any mtDNA examined.

The second category of *mss51* mutants, *-51* and *-52*, were Arg+ and Pet- in strains bearing *cox1Δ::ARG8^m cox2Δ::COX1*and *COX1(1-512)::ARG8^m* mtDNA, and were Pet- in strains bearing wild type mtDNA when on a plasmid (Figure 2.5). The mutant phenotypes of *mss51-51* and *mss51-52* were unchanged on a plasmid or integrated (Figure 2.6). *mss51-51* has two sets of mutations, three clustered missense mutations in the first third of the gene, and a frameshift insertion two thirds of the way through the gene. I initially attempted to determine if the 3 missense mutations or the frameshift mutation was responsible for the mutant phenotype of *mss51-51*, but never obtained integrated mutants that were either the missense mutations or the frameshift mutations alone. Efforts on *mss51-51* were put aside in favor of *mss51-52* because *mss51-52* showed a similar phenotype to *mss51-51* and I obtained an unlinked suppressor of *mss51-52*'s nonrespiratory phenotype.

The Arg+ phenotype of *mss51-51* and *mss51-52* in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA indicate that these mutants are able to activate translation at the *COX1* 5' UTR, although it is attenuated significantly. The cause of *mss51-52's* inability to respire in strains bearing wild type, *COX1(1-512)::ARG8^m* or *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA will be investigated in the next chapter.

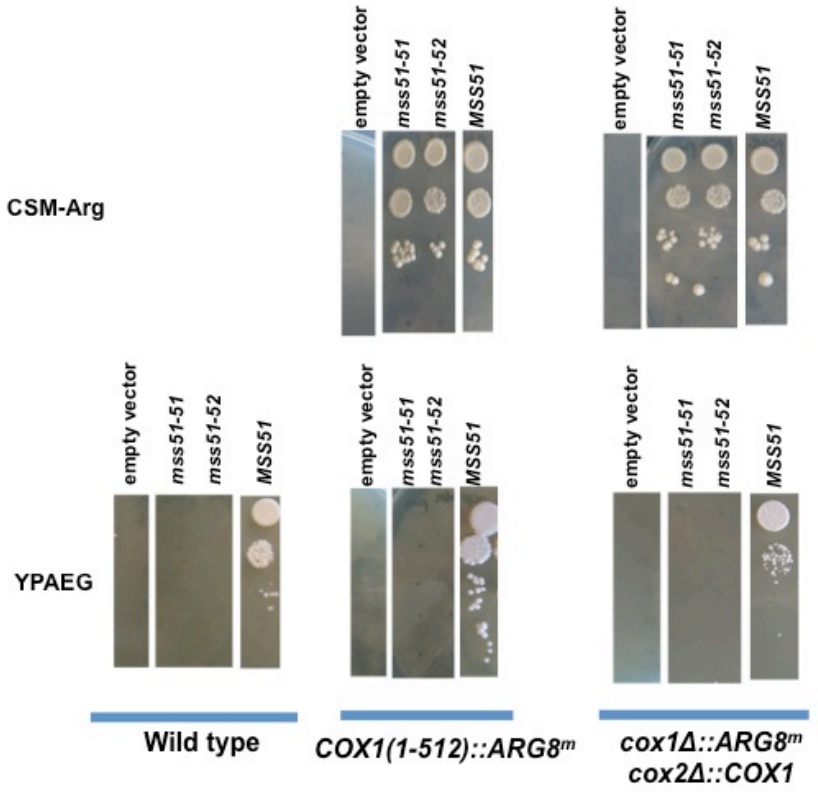

Figure 2.5 – Plasmid borne *mss51-51* and *mss51-52* are unable to support respiration. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA and containing *mss51* alleles on a ARS CEN vector were spotted medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. Strains bearing wild type mtDNA were: empty vector, XPM89 + pRS316; *mss51-51*, XPM89 + pZV2; *mss51-52*, XPM89 + pOAB2*; MSS51,* XPM89 + pCB16. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: empty vector, XPM76 + pRS316; *mss51-51*, XPM76 + pZV2; *mss51-52*, XPM76 + pOAB2*; MSS51,* XPM76 + pCB16. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were: empty vector, XPM174 + pRS316; *mss51-51*, XPM174 + pZV2; *mss51-52*, XPM174 + pOAB2*; MSS51,* XPM174 + pCB16. This dilution has not been repeated. See Table 2.4 for complete genotypes.

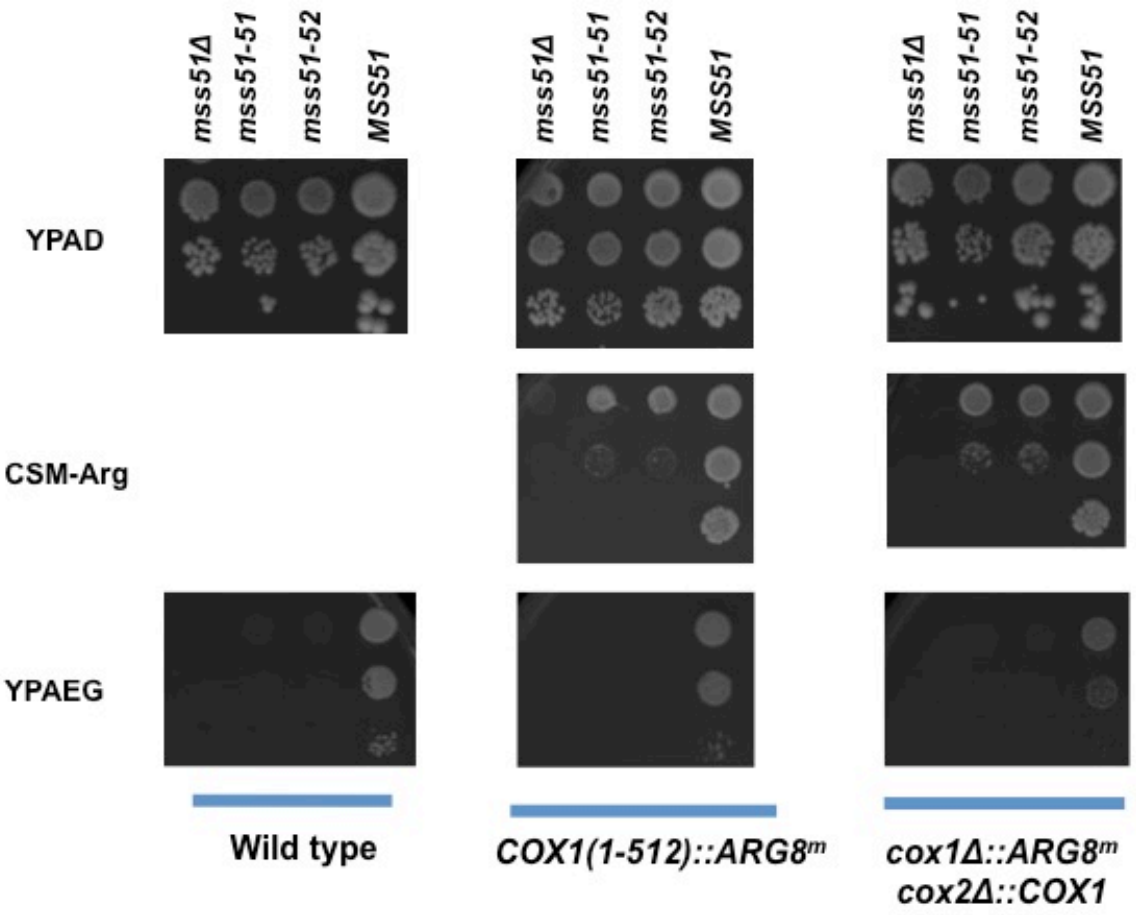

Figure 2.6 – Integrated *mss51-51* and *mss51-52* are unable to support respiration. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-51*, ZV17a; *mss51-52*, CAB322*; MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM76; *mss51-51*, ZV19a; *mss51-52*, ZV21a; *MSS51,* XPM78. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-51*, ZV8; *mss51-52*, ZV13a; *MSS51,* XPM171. See Table 2.4 for complete genotypes.

Summary of mutant screen

A strain bearing the *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA that Xochitl Perez-Martinez created proved quite successful in identifying separation of function *mss51* mutants that allow for translation activation at the *COX1* 5' UTR, but are unable to support respiration. The cause of the Pet- phenotype could be several possible factors – an inability of *mss51* to interact with nascent Cox1p or another Cox1p assembly factor, a failure of mutant mss51p to dissociate from the intermediate assembly complex, or just a general decrease in all these functions, which lower cytochrome *c* oxidase assembly to a level below the threshold necessary for respiration. In the next three chapters, two particular alleles will be discussed, *mss51-52* and *mss51-102*, and I will attempt to discern the cause of their inability to support respiration.

In addition to *mss51-51, -52, -102* and *-103*, which are the only mutants that have been sequenced, an additional 13 *mss51* mutants are Arg+ and Pet- in *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA and are frozen away as E. coli stocks as pZV30-pZV42. These mutants were initially identified in strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, isolated from yeast, transformed into E. coli, and retransformed into strain XPM174, where the initially observed phenotype was recapitulated. These mutants have not been sequenced or integrated, nor have their phenotypes been observed in strains bearing *COX1(1-512)::ARG8^m* or wild type mtDNA. If these mutants were to be pursued, I would suggest that integrating them into strain CAB305 is of the most immediate priority. As found with *mss51-101*,

plasmid versus integrated phenotypes are not necessarily the same, and integrated mutants are more biologically relevant than when on a plasmid.

I was unable to identify a domain or motif of *MSS51* based on the sequences of the mutants I examined. As only four *mss51* alleles were integrated, such an analysis would prove challenging due to lack of sufficient data. It might be worthwhile to sequence the additional plasmids I have frozen away, although integrating and crossing of these mutants into *COX1(1-512)::ARG8^{m}* and *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA would be time consuming. Regardless, the mutant *mss51-52* did ultimately provide biologically relevant information concerning a residue of *MSS51* involved in its interaction with Cox1p, and *mss51-102* and *-103* showed that WT Mss51 may act in *cis* for its targeting function between the *COX1* 5' UTR and the Cox1 protein.

ACKNOWLEDGEMENTS

One of the mutant screens was performed with the help of a summer rotation student Omer Ali Bayraktar. In addition to general lab assistance, Christine Butler helped significantly with the integration of the *mss51* alleles into CAB305.

Table 2.4 – *S. cerevisiae* strains used in Chapter 2		
Strain	**Genotype**	**Reference**
CAB322	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-52::3xHA, COX14::3xMYC* [ρ+]	This study
CAB323	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-101::3xHA, COX14::3xMYC* [ρ+]	This study
CAB324	MATa*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, COX14::3xMYC* [ρ+]	This study
CAB325	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-103::3xHA, COX14::3xMYC* [ρ+]	This study
CK520	MATa, *leu1, kar1-1, canR* [ρ+ ΔΣaI, ΔΣb]	(Labouesse , 1990)
DAU1	MATa, *ade2, ura3-Δ* [ρ+]	(Costanzo and Fox, 1988)
PTH60	MATα, *ura3-52, lys2-187* [ρ+]	(Das, 2006)
SB14a	MATa, *leu2-ΔClaI,EcoRV, ade2, ura3-52, mss51Δ::LEU2* [ρ+]	(Perez-Martinez, 2003)
XPM76	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM78	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM89	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+]	This study
XPM171	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
XPM174	MATα, l*ys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2*	(Perez-Martinez,

	[ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	2003)
ZV8	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-51::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV13a	MATa, *lys2, leu2-3,112, arg8::hisG, his3Δ::HindIII, ura3::kanMX3, mss51-52::3xHA, COX14::3xMYC* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV14a	MATα, *lys2, leu2-3,112, arg8::hisG, ura3::kanMX3, mss51-101::3xHA, COX14::3xMYC* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV17a	MATα, *lys2, leu2-3,112, ura3-52, mss51-51::3xHA* [ρ+]	This study
ZV18a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, COX14::3xMYC* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV19a	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-51::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV21a	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-52::3xHA, COX14::3xMYC* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV22a	MATα, *lys2, leu2-3,112, arg8::hisG, his3Δ::HindIII, ura3::kanMX3, mss51-103::3xHA, COX14::3xMYC* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV25a	MATa*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-103::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV26a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3::kanMX3, mss51-101::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV27a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study

All strains are congenic or isogenic to D273-10B, except CK520, PTH60 and SB14a

Mitochondrial genotypes are shown in brackets. ΔΣaI, ΔΣb refers to an intronless mitochondria derived from CK520

c Ectopic insertion of the chimeric COX1 upstream of COX2

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Chapter 3

mss51-W64R is the single missense mutation responsible for the nonrespiratory phenotype of *mss51-52*

INTRODUCTION

Based on the phenotypes shown in Figure 2.6, *mss51-52* appears to represent a separation-of-function *mss51* allele. This chapter covers initial characterization of the mutant allele *mss51-52* and the identification of a single nuclear dominant allele specific suppressor of the nonrespiratory phenotype of *mss51-52*. When chromosomally integrated in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, the phenotype of *mss51-52* is Arg+ and Pet-. The ability to support growth on CSM-ARG indicates that *mss51-52* is able to activate translation at the *COX1* 5' UTR, and the inability to support respiration means that *mss51-52* is defective in at least one step downstream of *COX1* translation activation.

METHODS AND MATERIALS

Strains, media and genetic methods

Saccharomyces cerevisiae strains used in this chapter are listed in Table 3.4**.** See Chapter 2 for media and genetic methods.

Western blot analysis

Total protein was prepared from cells grown in YPARaffinose media until mid log phase by extracting with NaOH and beta-mercaptoethanol (Yaffe and Schatz 1984). Crude mitochondria were prepared by growing cells in complete raffinose medium until mid log phase, and breaking the cells with glass beads (Diekert, de Kroon et al. 2001). Proteins were separated by SDS-PAGE on a 12% gel (Laemmli 1970). For anti-Arg8 westerns, proteins were transferred to Immobilon-P membrane (Immobilon) and western blots were probed with anti-Arg8 primary antibody (Steele, Butler et al. 1996), goat anti-rabbit HRP secondary antibody (Invitrogen), and detected with an ECL kit (GE Healthcare). For quantitative westerns, proteins were transferred to Immobilon-FL membrane (Immobilon) and western blots were probed with anti-HA primary antibody (Roche) and Alexo Fluor 488 IgG anti-mouse fluorescence secondary antibody (Invitrogen). The membrane was stripped with low pH buffer (500 mM NaCl, 100 mM Glycine, 0.5% Tween 20, pH 2.5), probed with anti-citrate synthase primary antibody (Schatz Lab), Alexo Fluor 488 IgG anti-mouse fluorescence secondary antibody. Band intensity was quantified on an AlphaImager Mutlimage III using FluorchemQ software (Cell Biosciences, Inc.). Units were calculated as anti-HA per anti-citrate synthase, and each anti-HA/anti-citrate

synthase was relative to *MSS51*-HA/*MSS51*-HA, which was set as a value of '1' for each experiment.

COX1 start codon mutation

Plasmid pZV8 was created by changing the *COX1* ATG start codon to ATC, with plasmid pXPM57 as a template, using the QuikChange mutagenesis kit (Stratagene). Plasmid pXPM57 contains intronless *COX1* with *COX1* flanked by 395 bp *COX1* 5' UTR and 990 bp *COX1* 3' UTR in pBluescript SK+ backbone. Primers used to change the ATG start codon into ATC were ZVCOX1QC F 5' TAA AAA AAA AGT AAA AAT CGT ACA AAG ATG ATT A 3' and ZVCOX1QC R 5' TAA TCA TCT TTG TAC GAT TTT TAC TTT TTT TTT A 3'. pZV8 was transformed into strain NAB69 rho0 by high velocity microprojectile bombardment (Bonnefoy and Fox 2001). Stable rho- transformants were selected by crossing the bombardment plates to M5-85 and selecting respiring diploids on YPAEG. M5-85 is a strain containing a nonfunctional *COX1* due to a mutation thought to be in the C terminal third of the gene (Bonitz, Coruzzi et al. 1980). 6 stable rho- *COX1* ATC were identified, called ZV35a-f. ZV35a-f were crossed to XPM11a in an attempt to find Arg(-) Pet(-) colonies, that became Arg(+) Pet(+) when crossed to 10A, a rho- strain that contains only *COX1* and flanking regions. A stable rho+ containing ATC in a strain bearing *COX1(1-512)::ARG8*^{m} mtDNA was never identified

mss51-52 Revertant Selection

mss51-52 on a plasmid was transformed into the strain SB14 (*mss51Δ::LEU2,* wild type mtDNA, DBY background) and the transformant failed to respire, as expected.

The strain was grown in CSM-URA liquid media to saturation and 200 uL was plated on YPAEG plates to select for respiring pseudorevertants.

To determine reversion rates of strains containing *mss51-52*, 10 independent colonies were selected from three strains – CAB322 (*mss51-52,* wild type mtDNA), ZV13a (*mss51-52, cox1Δ::ARG8^m cox2Δ::COX1* mtDNA) and SB14 + pOAB2 (*mss51-52* plasmid, wild type mtDNA, DBY background). Strains CAB322 and ZV13a were grown overnight to saturation in 5 mL YPAD, and the SB14 + pOAB2 was grown for 2 days in 5 mL CSM-URA. Initial number of cells in each culture was estimated using a hemacytometer (American Optical), and respiring pseudorevertants were scored after 7 days incubation at 30ºC.

Illumina/Solexa Sequencing

Genomic DNA of the parent (SB14) and suppressor (ZV80) strains was prepared using the 100/G Yeast Genomic Prep (QIAGEN), and submitted to the Cornell Core Facility for Illumina/Solexa sequencing. The sequence data was analyzed using the Maq software package by Stephan Stefanov and Qi Sun at the Core Facility. An annotated list of the top 9 nuclear and 1 mitochondrial mutation was returned as the most likely candidates.

Genomic Library Construction

The formation of the genomic library was performed according to the protocol of Rose (Rose and Broach 1991). A 100/G Yeast Genomic Prep (QIAGEN) of the ZV80 rho0 (suppressor) was performed. Initial digests with Sau3a (Invitrogen) were

performed with 1 ug suppressor gDNA and 1 U, 0.5 U, 0.25 U, 0.125 U, 0.00625 U, 0.0313 U, 0.0156 U, 0.0078 U Sau3a / ug suppressor gDNA. Digests were carried out at 37ºC for 1 hour, and quenched with 25 mM EDTA, 200 mM NaCl. The digests were visualized on a 0.4% agarose gel run at 30V for 5 hours.

Three scaled up digests with 100 ug suppressor gDNA each were digested with 0.125 U, 0.0875 U, and 0.0625 U Sau3a / ug suppressor gDNA, in a total volume of 1 mL. Digests were carried out at 37ºC for 1 hour, and quenched with 25 mM EDTA, 200 mM NaCl. The digests were visualized on a 0.4% agarose gel run at 40V for 5 hours. The three digests were pooled, phenol extracted, EtOH precipitated, and resuspended to a concentration of 1 ug / uL. The resuspended gDNA was then loaded on a 36 mL 10-40% sucrose gradient (Beckman) and spun 24 hour 26k rpm in a Beckman SW27 rotor. 10 drop fractions were collected and 1/25 volume of each was visualized on a 0.4% agarose gel run at 30V for 5 hours. Fractions containing 5-10 kb and 10-20 kb fragments were pooled separately. Each pool was dialyzed in 7000 MWC SnakeSkin dialysis tubing (Pierce) 3 x 1L TE pH 8.0, 8 hours per treatment. After dialysis, each pool was concentrated using *sec*-butanol to ~500 uL, EtOH precipitated and resuspended to a final volume of 30 uL in TE pH 8.0, and quantified.

The plasmid vector used was pNB34. pNB34 is a low copy shuttle plasmid containing *URA3* and confers ampicillin resistance in *E. coli* and *S. cerevisiae.* Selection of insert containing plasmids is made possible by digesting pNB34 with BclI, which has a site in the λ *cI* gene. The λ *cI* regulates expression of a kanamycin

resistance cassette, and plasmids lacking inserts should not grow on LB + Kan (Hamel, Lemaire et al. 1998; Williams, Perez-Martinez et al. 2004). pNB34 was grown in a dam- strain, as BclI activity is blocked by *dam* methylation, and prepared using an E. coli MIDI prep (QIAGEN). The vector was digested with BclI (Invitrogen) and treated with CIAP (Invitrogen). It was found that digesting pNB34 with 1 U BclI / ug plasmid and treating the digested vector with 1 U CIAP / ug plasmid were the best conditions for optimal ligation efficiency. Ligation conditions were 0.5 ug / 25 uL reaction, with a vector:insert ratio of 2:1, performed O/N at 16ºC. Ligations were electroporated into the most competent cells that could be acquired, which at the time were MegaX DH10B T1R (Invitrogen). Transformed cells were plated to LB+Kan. The approximate fraction of large colonies to small colonies was estimated, and 9 individual small colonies were picked from each transformation pool to determine average insert size. Colonies were washed from the LB+Kan plates using LB, and E. coli MIDI preps were performed on the concentrated colonies from each pool.

The 5-10 kb and 10-20 kb plasmid libraries were serially diluted to determine and transformed into 1 mL of saturated CAB322 grown in YPAD media. Post transformation, the cells were spun down and resuspended in SD + 1 M Sorbitol prior to plating on CSM-URA. I used the dilution that yielded approximately 1000 transformants per plate, when 200 uL of the SD + 1 M Sorbitol media was plated to each CSM-URA plate. The CSM-URA transformants were replica plated to YPAEG to select for respiring colonies, which were then tested for linkage of the plasmid to the respiratory phenotype.

Bulk Segregant Analysis

To generate a strain isogenic to the suppressor, parent SB14a was mated to PTH282, diploids were selected, sporulated and tetrads dissected. A Matalpha, Leu+, Pet- haploid was obtained. This haploid was mated to ZV80, sporulated, and tetrads were dissected. 40 Pet+ and 40 Pet- spores were obtained. Each was inoculated into 2 mL YPAD, and 200 uL of each Pet+ and Pet- were combined into a Pet+ and Pet- pool and genomic DNA was prepared using 20/G Yeast Genomic Prep (QIAGEN). A third genomic prep was performed on a 50:50 mixture of pooled Pet+:Pet- cultures. Primers S24F1 5' CAG TTT CAT ACT TAT TTA AAT G 3' and S24R1 5' GTT CTT TAT CCT ATG TGA TTC G 3' were used to PCR amplify and sequence tW(CCA)K from the pooled Pet+, Pet- and 50:50 Pet+:Pet- gDNA, as well as gDNA from the parent and suppressor.

Primers YIL169C F1 5' CAG CAA GCC TCA TCA TTG C 3' and YIL169C R1 5' GAA ATT GCA CTA GAA GCC TTG G 3' were used to amplify and sequence YIL169C, primers YDR082W F1 5' GTT GAT ATT CCA TTT CTG TGC G 3' and YDR082W R1 5' CAG GCC TAG AGA GAT GCG CCG 3' were used to amplify and sequence YDR082W, and primers ATP25 F1 5' GAA GAT TAA GTG TAA TAG CCT G 3' and ATP25 R3 5' GGC GAA CGA CGA GAG ATT AAG 3' were used to amplify and sequence YMR098C.

Plasmid construction

Plasmid pZV16a was created using QuikChange PCR mutagenesis (Stratagene) by introducing the missense mutation W64R into pCB16 with primers MSS51 QC T190C F 5' GAG AAC AGA TTC CAT CCA CGG GAT CAG TCT CCC TCA G' and MSS51

QC T190C R 5’ CTG AGG GAG ACT GAT CCC GTG GAT GGA ATC TGT TCT C 3’. Plasmid pZV17a was creating using QuikChange PCR mutagenesis (Stratagene) by removing the missense mutation W64R from pOAB2 using primers MSS51 QC C190T F 5’ GAG AAC AGA TTC CAT CCA TGG GGT CAG TCT CCC TCA G 3’ and MSS51 QC C190T R 5’ CTG AGG GAG ACT GAC CCC ATG GAT GGA ATC CTG TTC TC 3’.

RESULTS AND DISCUSSION

mss51-52p steady state levels are reduced to 75% of MSS51p

I wanted to determine the cause of the inability of *mss51-52* to support respiration in strains bearing either wild type, *COX1(1-512)::ARG8^{m}* or *cox1Δ::ARG8m cox2Δ::COX1* mtDNA. One possible explanation was that steady state Mss51-52p levels were decreased significantly relative to wild type. Ideally the Pet- phenotype would be caused by a function of the mutant protein, not a simple quantitative effect of steady state protein levels. Using quantitative fluorescence Westerns, Mss51-52-HAp was found to be reduced to approximately 75% of Mss51-HAp levels (Figure 3.1). As this reduction is not a dramatically significant decrease, I concluded that the nonrespiratory phenotype is likely not a cause of decrease in steady state protein levels.

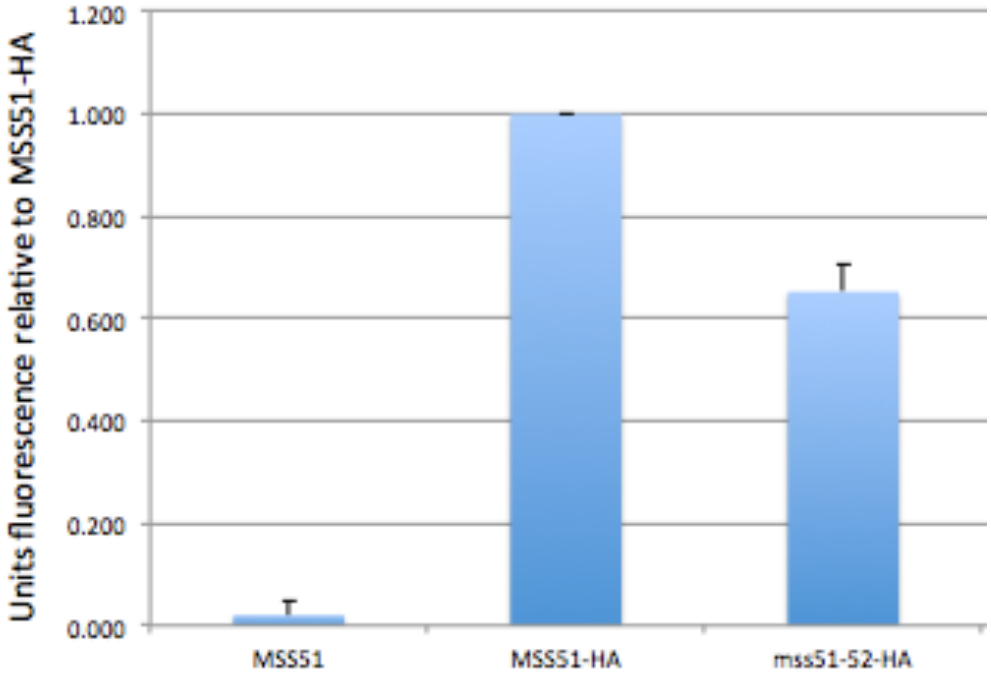

Figure 3.1 – *mss51-52* caused *Mss51-52-HA* protein levels to be reduced approximately 25%. 35 ug of total proteins was separated by 12.5% SDS-PAGE. Quantitative fluorescence Western blot analysis of total protein prepared from strains with the indicated relevant nuclear genotype was performed using anti-HA and anti citrate synthase antibodies. In each strain, units anti-HA per units anti-citrate synthase was determined, and normalized to *MSS51::3xHA,* which was set to 1 within each experiment. The solid bar represents the average and error bars are the range (N=3). Strains bearing wild type mtDNA were: *MSS51,* DAU1; *MSS51::3xHA,* SB7; *mss51-52::3xHA,* CAB322. See Table 3.4 for complete genotypes.

mss51-52 does not support steady state Cox1-Arg8^{m}p in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA

The *COX1(1-512)::ARG8^{m}* mtDNA construct created by Perez-Martinez provides a phenotypic reporter for *COX1* translation (Perez-Martinez, Broadley et al. 2003). An anti-Arg8 Western of a strain containing *MSS51* and bearing *COX1(1-512)::ARG8^{m}* mtDNA shows two distinct bands – an upper band around 102 kDa which is the Cox1p-Arg8p fusion protein, and a lower band of 44kDa which is Arg8p. Detection of the larger Cox1p-Arg8p band in an anti-Arg8 Western indicates that *MSS51* was able to promote full read through of the *COX1* coding sequence into the downstream reporter. That *mss51-52* is Arg+ but Pet- in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA is intriguing. When an anti-Arg8 western was performed on *mss51-52* in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA, the upper Cox1-Arg8m fusion protein was greatly reduced, if present at all, and instead a series of intermediate sized products were visualized (Figure 3.2). The level of mature Arg8 was also greatly reduced in the *mss51-52* mutant.

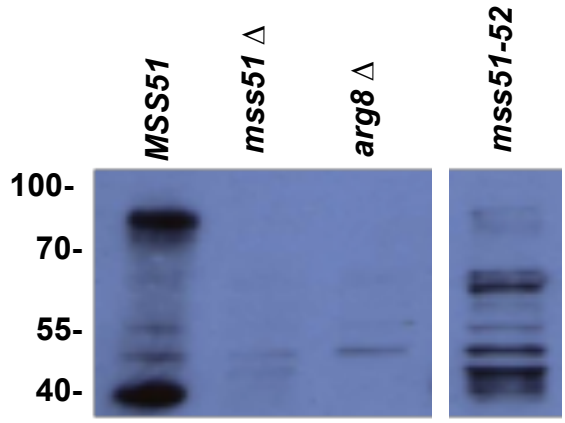

Figure 3.2 – *COX1(1-512)::ARG8*^{m} mtDNA provides a reporter for *COX1* translation. All strains bear *COX1(1-512)::ARG8*^{m} mtDNA. Relevant nuclear genotypes are as indicated. A 35 ug aliquot of a crude mitochondria preparation was separated by 12.5% SDS-PAGE, the Western blot was probed with anti-Arg8p antibody and visualized by ECL. Strains were: *MSS51,* XPM78; *mss51Δ,* XPM76; *arg8Δ,* NB40-30c; *mss51-52,* CAB322. This experiment has not been repeated. See Table 3.4 for complete genotypes.

The Arg+ phenotype means that *mss51-52* is somehow expressing *ARG8^m* downstream of *COX1* in the fused reporter construct. I considered two possibilities – either *mss51-52* was capable of translation activation at the *COX1* AUG start and complete read through was occurring but the Cox1 protein was unstable, or translation was initiating aberrantly at a downstream AUG codon within *COX1*. In order to test these two possibilities, I attempted to mutate the AUG start codon of *COX1* into ATC, which is previously described in *COX2* as nonfunctional start codon (Bonnefoy and Fox 2000). If translation activation was occurring at the normal AUG start and the Cox1p was unstable, a nonfunctional start codon would theoretically eliminate all *ARG8^m* translation. Alternatively, if translation activation was occurring at a downstream start site, mutating the normal AUG start codon would have little to no effect on the translation of *ARG8^m.*

An ATC start codon mutation was introduced into *COX1*, but despite a great deal of effort I was unable to create a stable rho+ *COX1(1-512)::ARG8^m* mtDNA with the start codon mutated. I was never able to figure out why I was unable to successfully introduce the start codon mutation into *COX1.* Despite this disappointment, the *COX1* start codon mutation ultimately proved to be unnecessary in regards to this project, due to the function of the single causative mutation responsible for the mutant phenotype of *mss51-52.* This will be discussed in Chapter 4.

Spontaneously Respiring Pseudorevertants of a strain containing *mss51-52* can be found at low frequency

I found that strains containing *mss51-52* were able to revert and gain the ability to support respiration. The estimated reversion percentage (number of revertants / total colonies) for ZV13a (*mss51-52*, *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, D273 background), CAB322 (*mss51-52,* wild type mtDNA, D273 background) and SB14 + pOAB2 (*mss51-52* on a plasmid, wild type mtDNA, DBY background) are shown in Table 3.1. Spontaneously respiring psuedorevertants occurred at approximately the same rate in the three nuclear and mitochondrial backgrounds tested, and appear to be unaffected by plasmid-borne compared to integrated *mss51-52*.

Table 3.1 – Reversion percentage of three strains bearing *mss51-52*

	Reversion percentage, mean of 10 independent colonies (# revertants / total colonies)
ZV13a	6.58E-08
CAB322	1.38E-08
SB14 + pOAB2	2.43E-08

MUT is a single dominant nuclear suppressor mutation, allele specific to *mss51-52*

In order to identify the missense mutation(s) responsible for the inability of *mss51-52* to support respiration, a spontaneously respiring pseudorevertant of plasmid borne *mss51-52* in SB14 was selected. The suppressor strain was named ZV80, containing the conditionally named mutation *MUT.* I isolated the plasmid containing *mss51-52* from ZV80, sequenced *mss51-52* from the plasmid and found that no additional mutations had occurred. In order to ascertain the mode of inheritance of *MUT*, ZV80 was crossed both as a rho+ and rho0 with CAB322 (*mss51-52,* wild type mtDNA) or CAB322 rho0, diploids were selected and Pet+ growth was examined. ZV80 is demonstrated to contain a nuclear dominant suppressor mutation, as both ZV80 and ZV80 rho0 allow a homozygous *mss51-52* diploid to respire (Figure 3.3A). ZV80 + pOAB2 was crossed to a CAB322 rho0 (*mss51Δ::LEU2,* rho0), diploids were selected, sporulated, and tetrads dissected on CSM-Ura to ensure that all spores contained a plasmid bearing *mss51-52*. The CSM-Ura plates were printed to YPAEG, and respiratory growth in the spores went 2:2, indicating that *MUT* is a single mutation (Figure 3.3B). *MUT* was found to be allele specific, as it was unable to suppress the nonrespiratory phenotype of *mss51-102* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA (Figure 3.4).

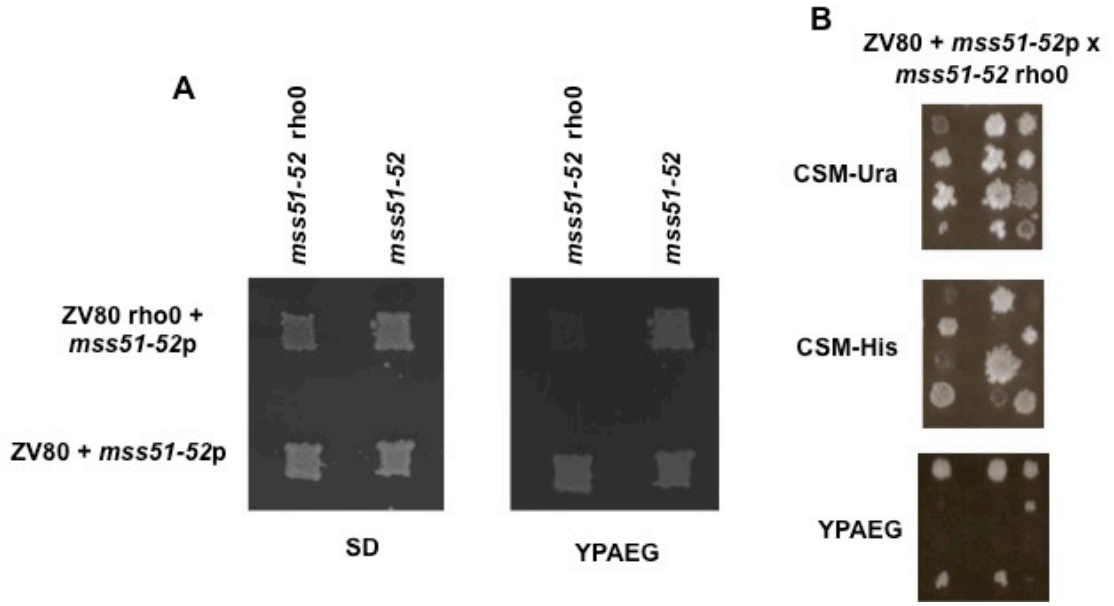

Figure 3.3 – *MUT* is a single nuclear dominant mutation that allows *mss51-52* to support respiration. (A) Haploid cells containing the relevant nuclear genotypes as indicated were patched in vertical stripes. *mss51-52*p is plasmid pOAB2. The stripes were cross-printed on YPAD medium and allowed to mate. Diploids were selected on synthetic deficient (SD) medium and printed to complete ethanol/glycerol (YPAEG) medium. Plates were incubated for 2 days at 30ºC. Strains were: ZV80 rho0 + *mss51-52*p, ZV80 rho0 + pOAB2; ZV80 + *mss51-52*p, ZV80 + pOAB2; *mss51-52* rho0, CAB322 rho0; *mss51-52,* CAB322. See Table 4 for complete genotypes. (B) Haploid cells containing the relevant nuclear genotypes as indicated were patched in vertical stripes. *mss51-52*p is plasmid pOAB2. The stripes were cross-printed on YPAD medium and allowed to mate. Diploids were selected, sporulated and tetrads were dissected on CSM-Ura. The tetrads were then printed to medium lacking histidine (CSM-His) and complete ethanol/glycerol (YPAEG) medium. Plates were incubated for 2 days at 30ºC. Figure 3.3A has been repeated once. See Table 3.4 for complete genotypes.

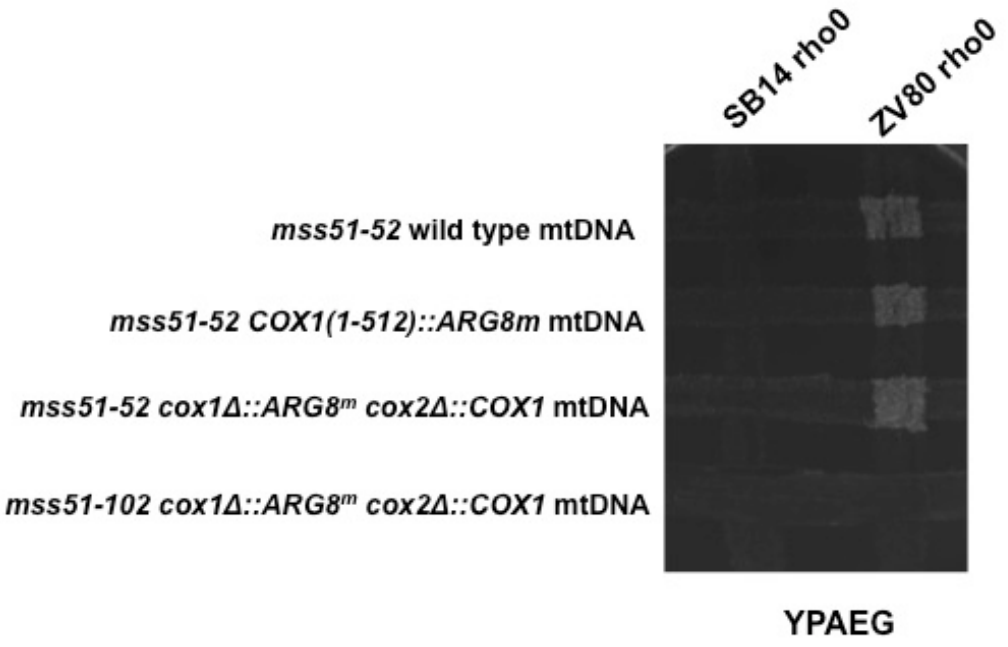

Figure 3.4 – *MUT* specifically suppresses the nonrespiratory phenotype of *mss51-52*. Haploid cells containing the relevant nuclear genotypes as indicated were patched in vertical stripes. The stripes were cross-printed on complete (YPAD) medium and allowed to mate. The plate was then printed to complete ethanol/glycerol (YPAEG) medium. Plates were incubated for 2 days at 30ºC. Strains were: *mss51-52* wild type mtDNA, CAB322; *mss51-52 COX1(1-512)::ARG8*^{m} mtDNA, ZV21a; *mss51-52 cox1Δ::ARG8*^{m} *cox2Δ::COX1 mtDNA*, ZV13a; *mss51-102 cox1Δ::ARG8*^{m} *cox2Δ::COX1 mtDNA,* ZV27a. This cross has been repeated once. See Table 3.4 for complete genotypes.

In order to determine if *MUT* was an informational suppressor, ZV80 was crossed to PTH60 (*MSS51, lys2-187*). *lys2-187* has been demonstrated to be an omnisuppressable allele (Das, Das et al. 2006). *MUT* failed to suppress *lys2-187* (data not shown), which suggested that *MUT* was not an informational suppressor, meaning that identification of *MUT* would be potentially worthwhile.

Illumina/Solexa sequencing analysis was used to identify *MUT*

Illumina/Solexa sequence analysis was initially utilized in an attempt to identify *MUT*. ZV80 and SB14 genomic DNA was submitted to the Cornell Life Sciences Core Laboratory Center. Computational analysis was performed by Stefan Stefanov and Qi Sun at the Core Center, and 8 putative nuclear mutations were identified (Table 3.2). One of the genes identified, *ATP25*, is involved in the stabilization of *ATP9*, and thus was an attractive potential candidate for *MUT*. Using targeted Sanger sequencing it was verified that the proposed T->A nucleotide substitution in *ATP25* was present in ZV80 and not in the parent strain SB14, which was an encouraging result (Figure 3.5). However, I needed to demonstrate that the mutation was linked to the respiring phenotype, and to accomplish this I used bulk segregant analysis.

Table 3.2 – Putative mutations in ZV80 identified by computational analysis.

chr	pos	refbase	mutation	readdepth	avhits	loglhrat	ORF
chr04	611294	C	G	14	0.5	190	YDR08W2
chr09	24287	G	T	85	0.69	97	YIL169C
chr09	207005	T		116	1.69	70	YIL082W-A
chr09	207007	A		115	1.62	83	YIL082W-A
chr11	302596	A	G	29	2.62	144	
chr11	578705	C	T	38	1.12	229	
chr11	635106	C	T	5	1	29	
chr13	461369	T	A	10	0.69	155	YMR098C
chr15	572103	C	T	5	1	1	
chrm	16540	T	C	255	0.62	69	Q0055

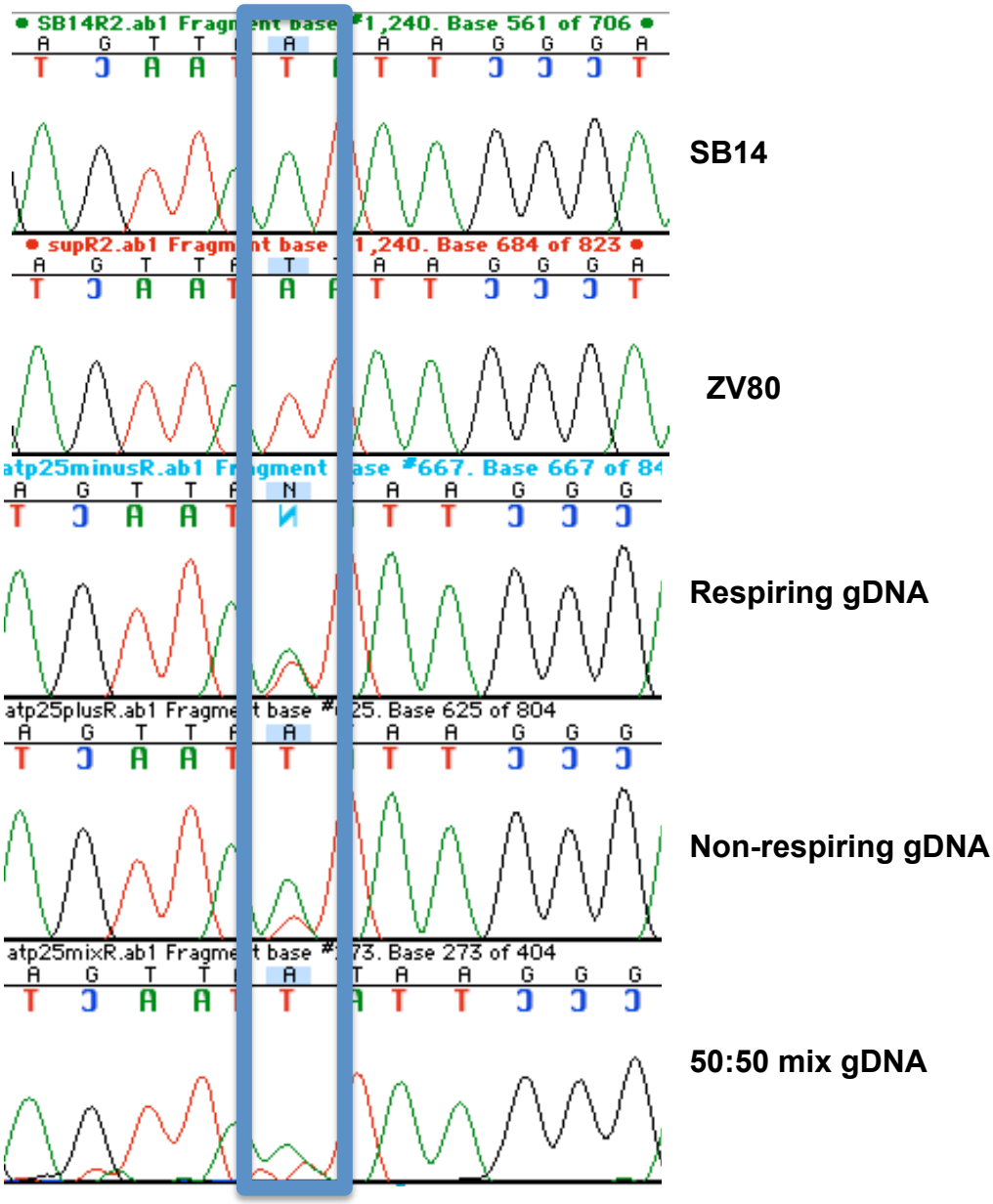

Figure 3.5 – The predicted mutation in *ATP25* is present in ZV80 but not genetically linked to the suppressor phenotype. This is an example of a mutation predicted by the Illumina/Solexa sequence that is present in ZV80, but segregates independently of the suppressor phenotype. See Table 3.4 for complete genotypes.

Bulk segregant analysis was used to link the suppressor mutation to the suppressor phenotype

Bulk segregant analysis is an efficient method that can be used to determine if a single mutation is linked to a phenotype (Brauer, Christianson et al. 2006). Bulk segregant analysis involves a cross two strains, one bearing a mutation that confers a phenotype (Figure 3.6). By examining the chromatograms of a single point mutation, it can be determined if a the mutation is genetically linked or unlinked to a phenotype.

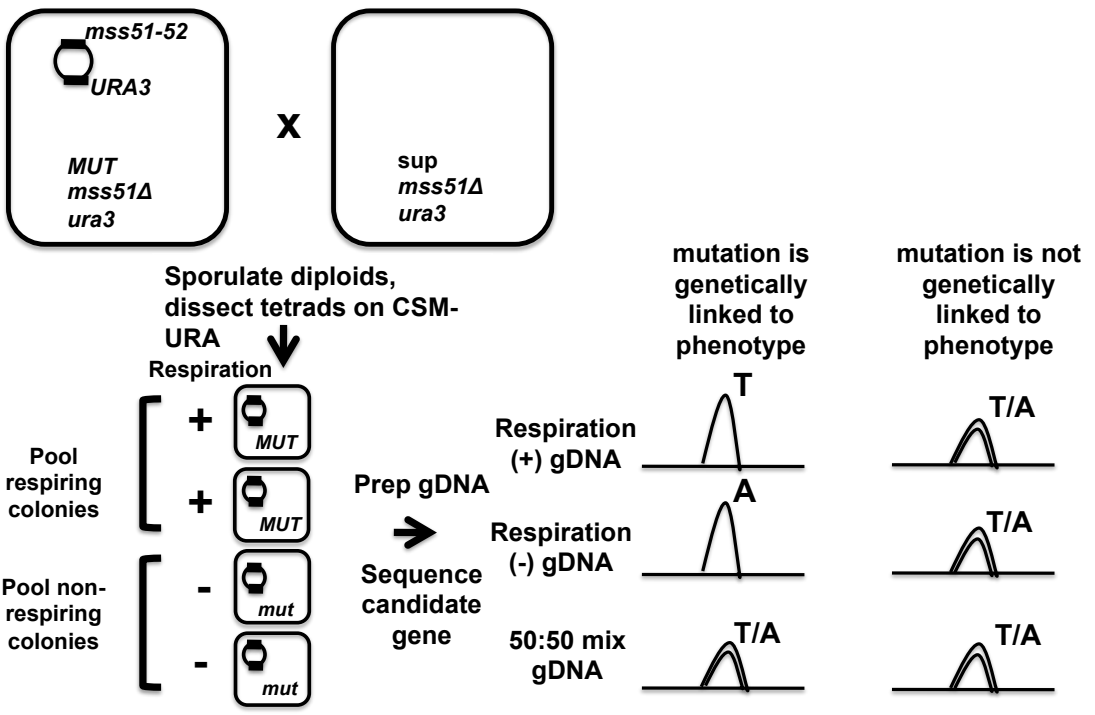

Figure 3.6 – Bulk Segregant Analysis can determine if a mutation is genetically linked to a phenotype. A visual schematic of bulk segregant analysis. Strains are crossed, one of which bears *mss51-52* on an ARS CEN vector. Diploids are selected, sporulated, and tetrads dissected on CSM-Ura to ensure the presence of the plasmid. Respiring colonies, which contain the *MUT* mutation, are pooled. Nonrespiring colonies, lacking the *MUT* mutation, are also pooled. Genomic DNA is prepared from each pool, and a candidate gene is sequenced from the respiring, nonrespiring, and 50:50 mix pool, and the chromatograms are examined from each pool. By analyzing the chromatograms of any candidate mutation, it can be determined if the mutation is linked or unlinked to the suppressor phenotype.

A pool of genomic DNA from 40 spores containing *mss51-52* and able to respire, thus containing the suppressor mutation, was created (see Methods and Materials). A second pool of genomic DNA from 40 spores containing *mss51-52* but lacking the suppressor mutation and therefore unable to respire was also created, as well as a third pool which consisted of a 50:50 mix of Pet+ : Pet- gDNA.

The three most likely candidate mutations in genes *STN1*, *ATP25* and *YIL169C* were analyzed via Sanger sequencing of the genomic pooled DNA from the bulk segregant analysis, and by examining the chromatograms all 3 mutations were found to assort independently of the respiratory phenotype, and thus were not *MUT* (Figure 3.5, and data not shown). As ATP25 was such an attractive candidate, linkage analysis using tetrads was performed, and I determined genetically that the mutation in ATP25 was unlinked to the suppressor phenotype (data not shown), which supported the Sanger sequencing results. It was remarkable that, at minimum, missense mutations in 3 separate protein coding genes accumulated between a parent and spontaneously respiring pseudorevertant. Unfortunately, *MUT* had not yet been identified.

A genomic library was created to clone *MUT*

As the top candidates from Illumina/Solexa sequencing were not responsible for the suppressor phenotype, I decided to make a genomic library of the nuclear dominant suppressor (see Methods and Materials). Two plasmid libraries were created, one containing 5-10 kb ZV80 gDNA fragments, and the other containing 10-20 kb ZV80 gDNA fragments. Summary statistics from the genomic library are listed in Table 3.3. Colonies growing on the LB + Kan plates were typically pencil-tip sized, with the occasional colony significantly larger. It was assumed that the larger colonies were ones that managed to grow on the LB + Kan plates without containing an insert, while the small colonies were thought to contain inserts. Plasmid DNA was prepared from pooled KanR *E. coli* colonies. When the plasmid pools were transformed into CAB322 (*mss51-52*, wild type mtDNA), 3 plasmid-linked respiring transformants from the 5-10 kb fragment pool were found, and 15 plasmid-linked respiring transformants from the 10-20 kb fragment pool were identified. A plasmid was isolated from one respiring transformant from each pool, and the inserts were sequenced. Both plasmids contained a piece of DNA around position 302,000 of Chr XI. When aligned to the chromosomal DNA, each plasmid contained an A -> G mutation at position 302,596, which corresponds to the 35th nucleotide of the Trp tRNA tW(CCA)K. This nucleotide is the third anticodon base. The mutation should enable the mutant Trp tRNA to recognize the CGG arginine codon.

Table 3.3 – Summary statistics from genomic library construction.

Pool	Total Colonies in *E. coli*	Approximate fraction of large colonies in *E. coli*	Small colonies with inserts (out of 9) in *E. coli*	Average insert size (kb)	Potential Respiring Transformants in *S. cerevisiae*	Verified Respiring Transformants in *S. cerevisiae*
5 - 10 kb	320000	1/150	8/9	4.9	17	3
10 - 20 kb	556000	1/200	9/9	13.6	59	15

An A->G mutation in the anticodon of tW(CCA)K is linked to the respiratory phenotype

It was found that the parent contained a G nucleotide at base pair 35, while the suppressor contained an A. Bulk segregant analysis was performed as previously described, examining the putative mutation in Trp tRNA tW(CCA)K. The chromatograms of the mutation G35A were examined from the pooled genomic samples and it was found that the Pet+ pool had A, the Pet- pool had G, and 50:50 mixture had A+G peak of equal intensity at the same position, demonstrating linkage of the G35A mutation to respiration (Figure 3.7). The A->G mutation in Trp tRNA tW(CCA)K was positively identified as *MUT.*

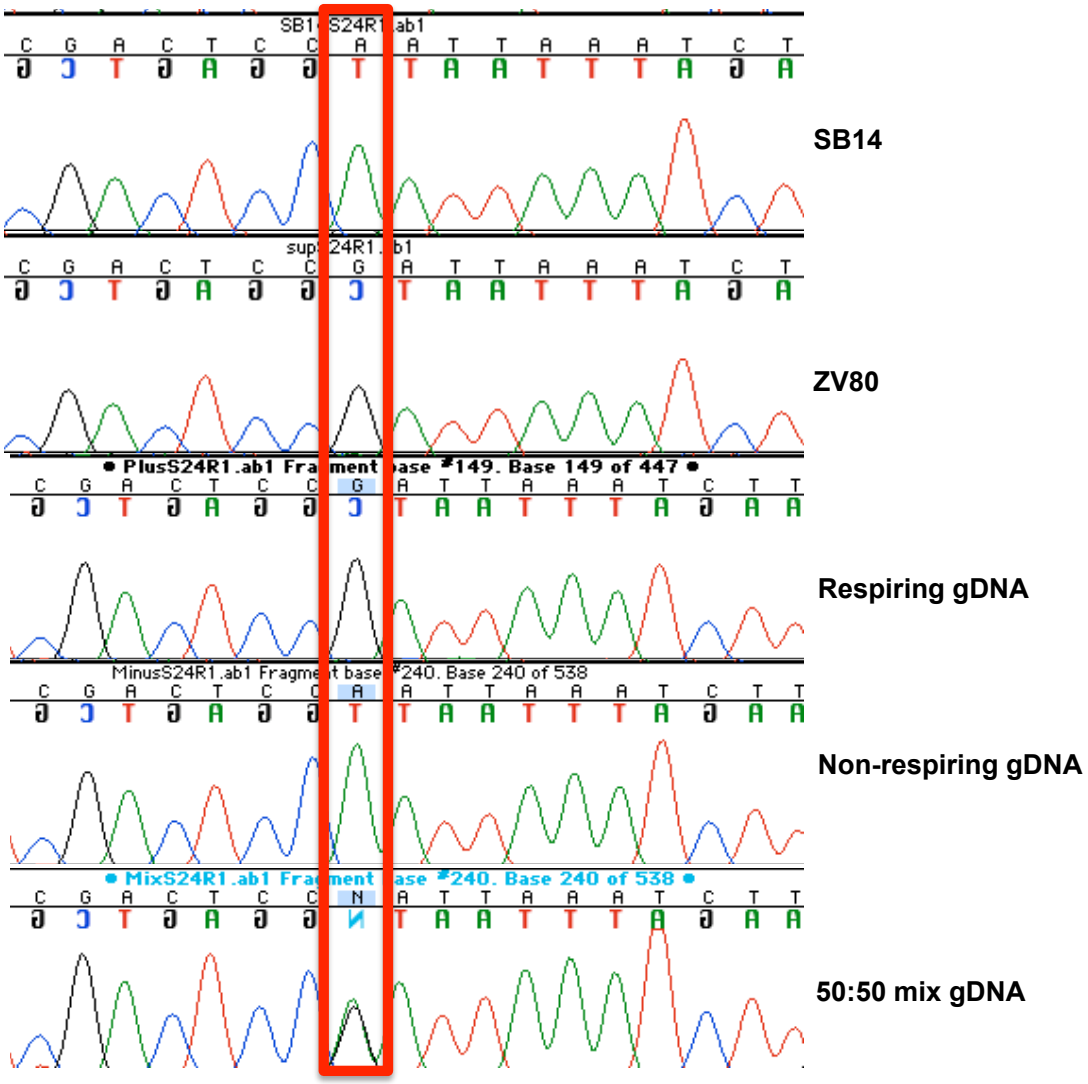

Figure 3.7 – The predicted mutation in *tW(CCA)K* is present in ZV80 and genetically linked to *MUT*. This is an example of a mutation predicted by the Illumina/Solexa sequence that is present in ZV80, and is linked to the suppressor phenotype. This sequencing has been repeated once. See Table 3.4 for complete genotypes.

When I looked back at the top hits from the Illumina/Solexa sequencing data, I found that the tW(CCA)K mutation was indeed identified by the computational analysis (chr11, position 302596). However, only the protein coding genes were annotated, so I disregarded the mutation as a viable candidate. It is unfortunate that I failed to identify the mutation prior to the creation of the genomic library, but it is comforting that the Illumina/Solexa sequencing and bulk segregant analysis support the conclusions of the genomic library.

mss51-W64R is Arg+ and Pet- in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA

The identity of *MUT* immediately suggested that the missense mutation W64R in *mss51-52* might be the causative mutation of lack of respiration. The W64R mutation was introduced into otherwise wild type *MSS51::3xHA* with QuikChange (Stratagene) creating the allele *mss51-W64R*, which is in plasmid pZV16a*. mss51-W64R* from pZV16a was integrated into CAB305, made rho0 via 2x EtBr treatment, and crossed into strains bearing *COX1(1-512)::ARG8^m* and *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA. In a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, *mss51-W64R* recapitulated the Arg+, Pet- phenotype of *mss51-52*, so it was concluded that *W64R* was the causative mutation of the lack of respiration of *mss51-52* (Figure 3.8). I also created and integrated the allele *mss51-54* using QuikChange*,* which is the reciprocal allele to *mss51-W64R. mss51-54* restores *mss51-52's* 64th amino acid residue back to tryptophan and retains the additional 5 missense mutations, in plasmid pZV17*. mss51-54* supports respiratory growth (Figure 3.8). The phenotypes of *mss51-W64R*

and *mss51-54*, as well as genetic and biochemical analysis of *mss51-W64R,* will be discussed in the next chapter.

ACKNOWLEDGEMENTS

Christine Butler performed the necessary crosses, tetrad dissections and DNA preparations to create the pooled Pet+ and Pet- genomic DNA analyzed in the bulk segregant analysis. Computational work on the Illumina/Solexa sequencing was performed by Stephan Stefanov and Qi Sun at the Cornell Life Science Core Laboratories Center.

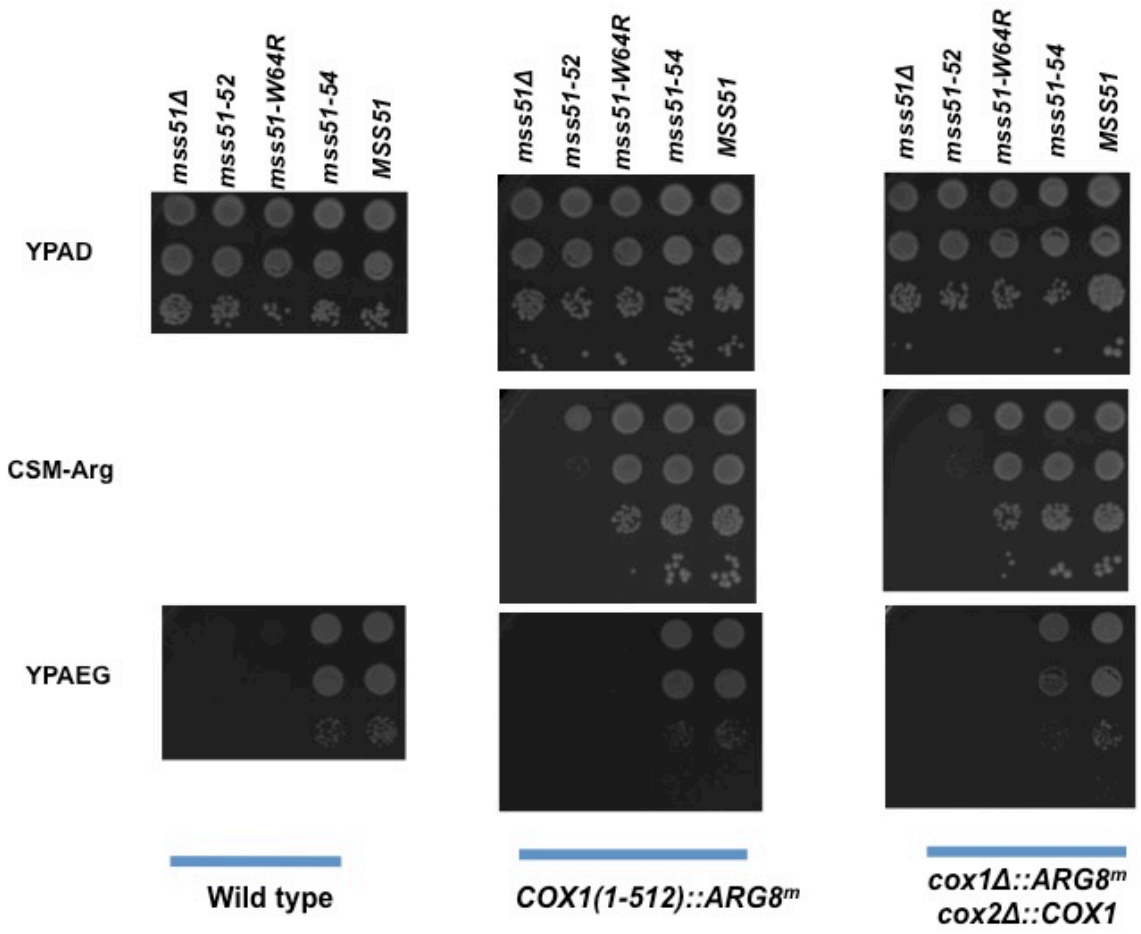

Figure 3.8 – *mss51-W64R* is unable to support respiration, while *mss51-54* is able to support respiration. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-52*, CAB322*; mss51-W64R,* ZV43, *mss51-54,* ZV40; *MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM76; *mss51-52*, ZV21a; *mss51-W64R,* ZV50a, *mss51-54,* ZV41a; *MSS51,* XPM78. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-52*, ZV13a; *mss51-W64R,* ZV48a; *mss51-54,* ZV37a; *MSS51,* XPM171. This dilution series has been repeated once. See Table 3.4 for complete genotypes.

Table 3.4 – *S. cerevisiae* strains used in Chapter 3		
Strain	**Genotype**	**Reference**
CAB322	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-52::3xHA, COX14::3xMYC* [ρ+]	This study
DAU1	MATa, *ade2, ura3-Δ* [ρ+]	(Costanzo and Fox, 1988)
M5-85	MATα [ρ-, COX1 point mutation]	(Bonitz, 1980)
NAB69 rho0	MATa, *ade2-101, arg-Δ::hisG, ura3-52, kar1-1* [ρ0]	(Perez-Martinez, 2003)
NB40-36a	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+]	(Perez-Martinez, 2003)
PTH282	MATα, *ade2-101, ura3-52, leu2-Δ* [ρ+]	This study
SB14a	MATa, *leu2-ΔClaI,EcoRV, ade2, ura3-52, mss51Δ::LEU2* [ρ+]	(Perez-Martinez, 2003)
SB7	MATα, *ade2, ura3-Δ, MSS51::3xHA* [ρ+]	(Perez-Martinez, 2003)
XPM76	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM78	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM89	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+]	This study
XPM11a	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study

XPM171	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
XPM174	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
ZV13a	MATa, *lys2, leu2-3,112, arg8::hisG, his3Δ::HindIII, ura3::kanMX3, mss51-52::3xHA, COX14::3xMYC* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV35 a-f	MATa, *ade2-101, arg-Δ::hisG, ura3-52, kar1-1* [ρ-, pZV8]	This study
ZV37a	MATa*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-54::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV40a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-54::3xHA* [ρ+]	This study
ZV41a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-54::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV43	MATa, *lys2, leu2-3,112, arg8::hisG, ura3::kanMX3, mss51-W64R::3xHA, COX14::3xMYC* [ρ+]	This study
ZV48a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV50a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV80	MATa, *leu2-ΔClaI,EcoRV, ade2, ura3-52, mss51Δ::LEU2*, *SUP* [ρ+]	This study

All strains are congenic or isogenic to D273-10B, except ZV80 and NAB69 rho0

Mitochondrial genotypes are shown in brackets. ΔΣaI, ΔΣb refers to an intronless mitochondria derived from CK520

c Ectopic insertion of the chimeric COX1 upstream of COX2

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Chapter 4

mss51-W64R is capable of activating translation at the *COX1* 5' UTR and is selectively deficient at a process downstream of *COX1* translation activation

INTRODUCTION

Cytochrome c oxidase (complex IV) is the terminal electron acceptor of the electron transport chain. The process of proper cytochrome oxidase assembly is complex and highly regulated, involving a host of mitochondrial- and nuclear-encoded proteins (Mick, Fox et al. 2011). The mitochondrially encoded *COX1* is the largest subunit of cytochrome *c* oxidase (Gray 2004). Proper assembly of Cox1 into cytochrome *c* oxidase is critical both to produce a functioning respiratory complex, and to protect the cell from oxidative damage (Khalimonchuk, Bestwick et al. 2009). Cox1 is highly hydrophobic, containing 12 transmembrane domains (Tsukihara, Aoyama et al. 1996), and is inserted into the inner mitochondrial membrane by the translocase Oxa1 (Hell, Neupert et al. 2001).

Translational activation of *COX1* occurs via the *COX1* 5' UTR by the nuclear encoded factors *MSS51* and *PET309* (Manthey and McEwen 1995; Perez-Martinez, Butler et al. 2009). *MSS51* is distinct among other *S. cerevisiae* mitochondrial translational activators in that it has an additional function, in its physical interaction with newly synthesized Cox1p, likely through the C-terminal residues (Perez-Martinez, Broadley et al. 2003; Shingu-Vazquez, Camacho-Villasana et al.

2010). Mss51 also has been found in cytochrome *c* oxidase intermediate assembly complexes with Cox14, Shy1, Coa3 and Coa1 (Barrientos, Zambrano et al. 2004; Mick, Wagner et al. 2007; Pierrel, Bestwick et al. 2007; Mick, Vukotic et al. 2010). Mss51 is involved in a dynamic regulatory pathway; it is thought to be sequestered in its intermediate assembly complexes by Cox14 and Coa3 which would prevent it from activating new *COX1* translation (Perez-Martinez, Broadley et al. 2003; Barrientos, Zambrano et al. 2004; Perez-Martinez, Butler et al. 2009; Mick, Fox et al. 2011). Both *coa3Δ cox14Δ* mutant are unable to respire but translate increased levels of *COX1* relative to WT, suggesting that *MSS51* is activating *COX1* translation at an increased rate (Barrientos, Zambrano et al. 2004; Mick, Vukotic et al. 2010). The manner of release of Mss51 from the Cox1 assembly intermediate is also of debate, although it has been proposed that Shy1 might cause Mss51 to dissociate, although others suggest nuclear assembly intermediates Cox5 or Cox6 might be involved (Barrientos, Zambrano et al. 2004; Shingu-Vazquez, Camacho-Villasana et al. 2010; Mick, Fox et al. 2011).

There are several published models of *MSS51* function (Barrientos, Zambrano et al. 2004; Mick, Wagner et al. 2007; Pierrel, Bestwick et al. 2007; Fontanesi, Soto et al. 2009; Perez-Martinez, Butler et al. 2009; Shingu-Vazquez, Camacho-Villasana et al. 2010; Mick, Fox et al. 2011). While some of these models differ in which cytochrome *c* oxidase assembly factors are physically associated with the Mss51-Cox1 assembly intermediate – Shy1 is of particular note – most are in agreement that Mss51-Cox1-Cox14-Coa1-Coa3 physically interact in a cytochrome *c* oxidase assembly intermediate. These models predict that Mss51 is physically

sequestered in this intermediate, thus becoming unavailable to initiate new Cox1 translation. The manner of release of Mss51 from the Cox1 assembly intermediate is also of debate, although it has been proposed that Shy1 might cause Mss51 to dissociate, although others suggest nuclear assembly intermediates Cox5 or Cox6 might be involved (Barrientos, Zambrano et al. 2004; Shingu-Vazquez, Camacho-Villasana et al. 2010; Mick, Fox et al. 2011).

This chapter will propose that *MSS51* has three distinct functions – translation activation at *COX1* 5' UTR, complete read through of the *COX1* message, and assembling Cox1p into cytochrome *c* oxidase. I have identified the mutant *mss51-W64R* that is able to activate *COX1* translation through the *COX1* 5' UTR and read through the full *COX1* message but is unable to respire, thus providing the first described separation-of-function *mss51* mutant.

METHODS AND MATERIALS

Strains, media and genetic methods

Saccharyomyces cerevisiae strains used in this chapter are listed in Table 4.1. See Chapter 2 for media and genetic methods.

Yeast transformation

See Chapter 2 for yeast transformation methods.

Western blot analysis

See Chapter 3 for Western blot analysis methods.

In vivo labeling of cycloheximide treated cells

In vivo pulse labeling of cells with [^{35}S] methionine was performed as previously described (Bonnefoy, Bsat et al. 2001; Perez-Martinez, Broadley et al. 2003). Crude mitochondria were prepared as described in Chapter 2. The radiolabeled proteins were separated by 12.5% SDS-PAGE and subjected to autoradiography.

Plasmid construction

Plasmid pZV18 contains *MSS51::3xHA* digested from pCB16 with *HindIII* and *XbaI*, and cloned into vector YEp352 using *HindIII* and *XbaI*. Plasmid pZV19 contains *MSS51::3xHA* digested from pZV16 with *HindIII* and *XbaI*, and cloned into vector Yep352 using *HindIII* and *XbaI*. pZV16 is *mss51-W64R::3xHA* in pRS316 vector, and pCB16 is *MSS51::3xHA* in pRS316 vector (see Chapter 2 Methods and Materials for

pCB16 construction, and see Chapter 3 Methods and Materials for pZV16 construction).

Preparation and solubilization of differential centrifugation purified mitochondria, and preparation of a sucrose gradient

Mitochondrial preparation

Cells were grown in 1 L 2% galactose, 1% yeast extract, 2% peptone medium at 30ºC to late exponential phase. Cells were pelleted 5 min 3000 x g, washed with 1.2 M sorbitol, centrifuged 3000 x g 10 min, and resuspended in digestion buffer (1.5 M sorbitol, 75 mM phosphate pH 7.5, 1.25 mM EDTA, 1.25% b-mercaptoethanol , 50 mg Zymolyase 20000) 10 g cells wet weight per 30 mL digestion buffer. All subsequent spins were performed in a Beckmann SS-34 rotor. Cells were incubated at 37ºC until spheroplasted. Equal volume of 1.2 M sorbitol, 20 mM KPO4 pH 7.5 was added and centrifuged 6200 rpm 10 min. Spheroplasts were washed twice with 1.2 M sorbitol, 20 mM KPO4 and resuspended in 0.6 M sorbitol, 20 mM KMES pH 6.0 0.5 mM PMSF at a concentration of 10 g spheroplasts wet weight / 30 mL. Spheroplasts were homogenized with 5-10 strokes of a Dounce homogenizer, spun at 3600 rpm 5 minutes, and the supernatant was collected. The spheroplast pellet was resuspended, homogenized and spun again, repeating the prior step. Both supernatants were combined and spun at 11.7k rpm 10 min to pellet mitochondria. The mitochondrial pellet was washed in 2 mL 0.6 M sorbitol, 20 mM KMES, diluted in 20 mL 0.6 M sorbitol, 20 mM HEPES and centrifuged 11.7 k rpm 10 minutes. The pellet was resuspended in 1 mL 0.6 M sorbitol, 20 mM HEPES and quantitated via

Lowry assay. 4 mg aliquots were prepared, snap frozen in liquid nitrogen, and stored at -80ºC.

Solubilization

4 mg differential centrifugation mitochondria were pelleted and resuspended in 400 uL extraction buffer (1% digitonin, 1.2 mM $MgCl_2$, 20 mM HEPES pH 7.4, 150 mM KCl, 0.5 mM PMSF, 1 mini protease inhibitor tablet (Roche)), and solubilized on ice with agitation for 30 minutes. Using a Beckman 50 Ti rotor, the mitochondria were spun for 23.6 k rpm for 15 minutes, after which the supernatant extract was promptly removed.

Sucrose gradients, Western blot analysis

5 mL 7-20% sucrose gradients were prepared in SW28 tubes (Beckman). The supernatant extract was carefully layered on the gradient, which was spun 12 hours 28000 rpm using a Beckman 55 Ti rotor. 14 equal fractions (approximately 350 uL each) were collected from the gradient, and 100 uL from each fraction was precipitated using Strataclean resin (Stratagene). All Strataclean-prepared fractions were separated on 12% SDS-PAGE, transferred to Immobilon-FL membrane (Immobilon), probed with an anti-MSS51 antibody and an anti-rabbit A488 fluorescent secondary antibody (Invitrogen), and visualized using an AlphaImager Mutlimage III with FluorchemQ software (Cell Biosciences, Inc.).

RESULTS

The single point mutation *mss51-W64R* results in Arg+ and Pet- growth in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA

The phenotype of the *mss51-W64R* allele is Arg+ and Pet- in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA (Figures 3.8 and 4.1). The ability of *mss51-W64R* to support growth on media lacking arginine is less strong than *MSS51*, indicating that it has decreased efficiency of translation activation at the *COX1* 5' UTR. *mss51-W64R* is able to support growth on media lacking arginine in a strain bearing *COX1(1-512)::ARG8^m* mtDNA but is unable to support respiratory growth. This suggests that full read through of the upstream *COX1* message is occurring. In a strain bearing wild type mtDNA, *mss51-W64R* fails to support respiratory growth. At endogenous levels, *mss51-W64R* is unable to support respiratory growth in any mtDNA I examined.

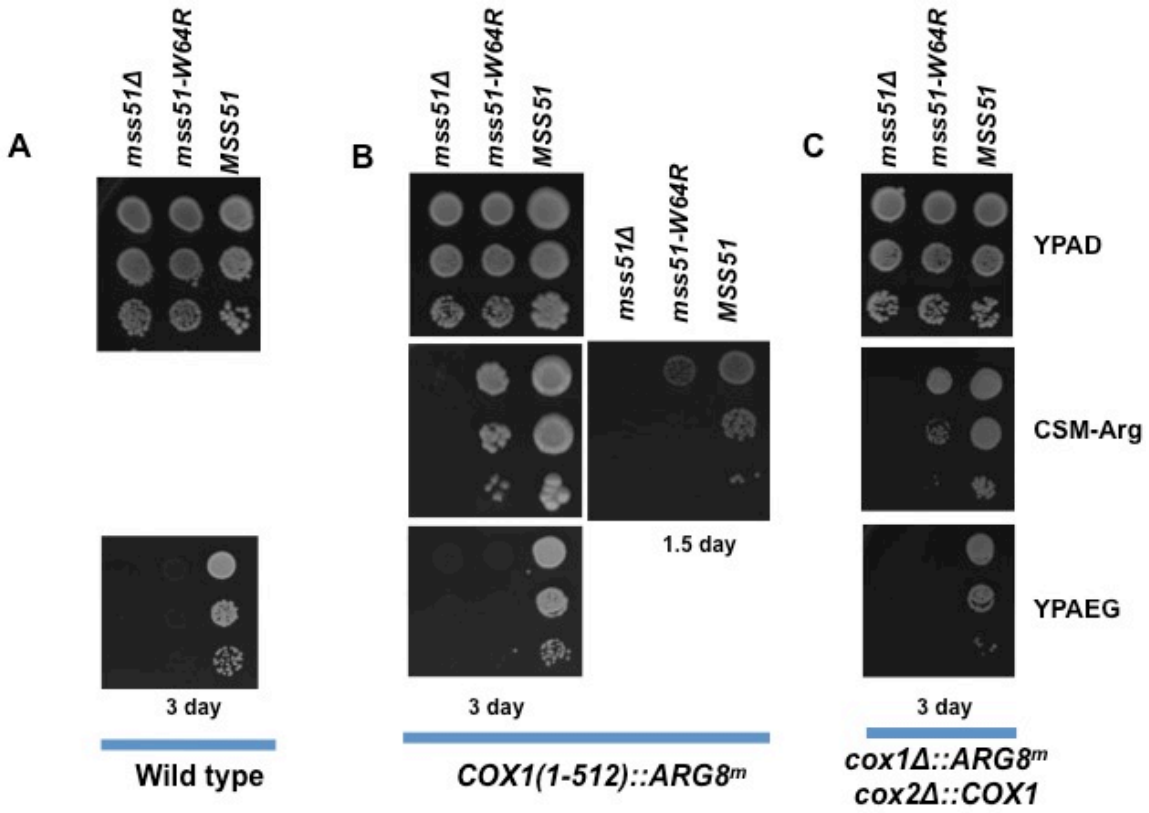


Figure 4.1 – *mss51-W64-HA* is a separation of function *MSS51* mutation that can activate translation at the *COX1* 5' UTR but is cannot support respiratory growth. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8^m* or *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 1.5 or 3 days at 30ºC as indicated. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-W64R*, ZV43; *MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8^m* mtDNA were: *mss51Δ*, XPM76; *mss51-W64R,* ZV50a; *MSS51,* XPM78. Strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-W64R,* ZV48a; *MSS51,* XPM171. This dilution series has been repeated once. See Table 4.1 for complete genotypes.

In an attempt to identify an interacting factor that when overexpressed allows for respiration when *mss51-W64R* is at endogenous levels, I obtained the Yeast Genomic Tiling Collection Pooled DNA from OpenBiosystems and transformed the collection into a strain containing *mss51-W64R* and bearing wild type mtDNA (Jones, Stalker et al. 2008). In addition to expected plasmids containing *MSS51*, we found plasmids containing WT copies of various Trp tRNAs were weakly able to suppress the inability of *mss51-W64R* to support respiratory growth. A similar phenomena has been previously described, and we did not pursue this further (Navarro and Thuriaux 2000).

The *mss51-W64R* is not completely deficient in its ability to support respiratory growth, however the lack of respiration is not due to a quantitative effect

I asked if *mss51-W64R* was completely deficient in its ability to support respiratory growth by overexpressing *mss51-W64R* on a 2 micron plasmid. When *mss51-W64R* was overexpressed in strains bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA, respiration was restored in all three mtDNA backgrounds, albeit to a level less that *MSS51* overexpressed on a 2 micron plasmid (Figure 4.2). This demonstrates that *mss51-W64R* is not completely deficient in its ability to support respiration.

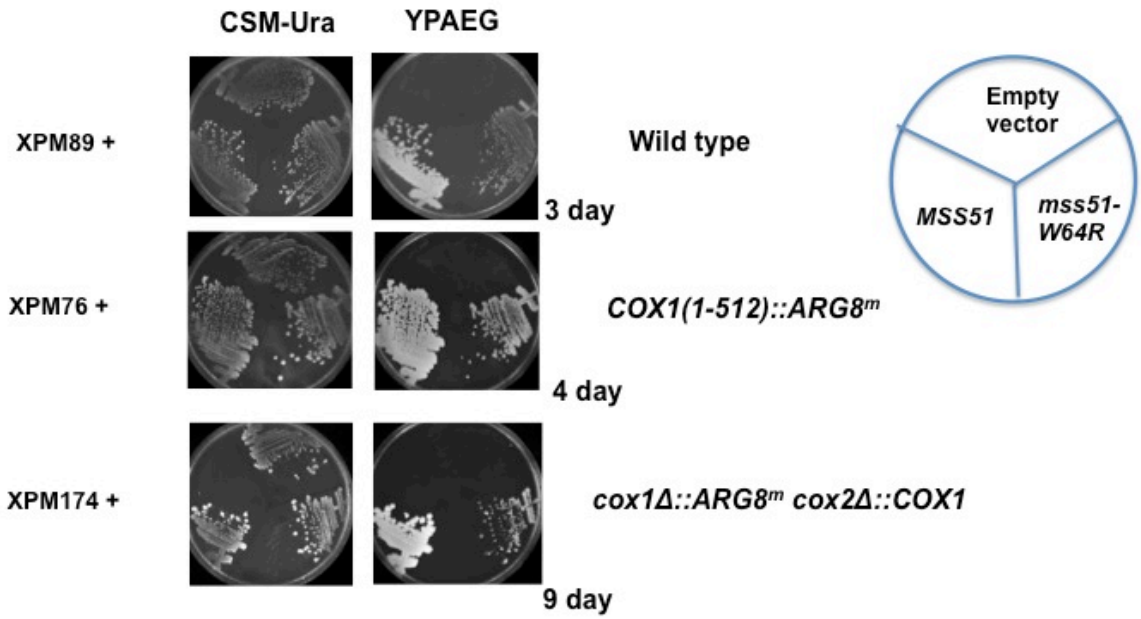


Figure 4.2 – *mss51-W64R* supports respiratory growth when overexpressed. Strains with *mss51Δ* and bearing either wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were transformed with either the 2u vector Yep352, pZV18 or pZV19. Strains were streaked to medium lacking uracil (CSM-Ura) to select for the plasmid, and printed to YPAEG medium to assess respiration. CSM-Ura were incubated for 3 days at 30ºC and YPAEG plates were incubated for 3, 4 or 9 days at 30ºC as indicated. This experiment has been repeated twice. See Table 4.1 for complete genotypes.

Once it was determined that *mss51-W64R* could support respiration when overexpressed on a 2u vector, we asked if *mss51-W64R*'s inability to support respiration at its endogenous loci was due to a quantitative reduction in steady state protein levels. A quantitative fluorescence Western blot analysis was performed on diploid strains containing (*mss51Δ/mss51Δ)*, (*mss51-W64R::3xHA/mss51Δ)*, (*MSS51::3xHA/mss51Δ), (mss51-W64::3xHA/mss51-W64R::3xHA),* and *(MSS51::3xHA/MSS51::3xHA)* (Figure 4.3). Heterozygous *mss51-W64R-HA* shows levels reduced to 33% of WT, yet is weakly Arg+ and unable to support respiratory growth. Both heterozygous *MSS51-HA* and homozygous *mss51-W64R-HA* have levels reduced to 60% of WT and have relatively equal levels of growth on media lacking arginine, yet *MSS51-HA/mss51Δ* is able to support respiratory growth, while *mss51-W64R-HA/mss51-W64R-HA* is unable to support respiratory growth. These data suggest the 40% reduction in steady state protein levels caused by *mss51-W64R-HA* in a diploid are not sufficient to cause the lack of respiration.

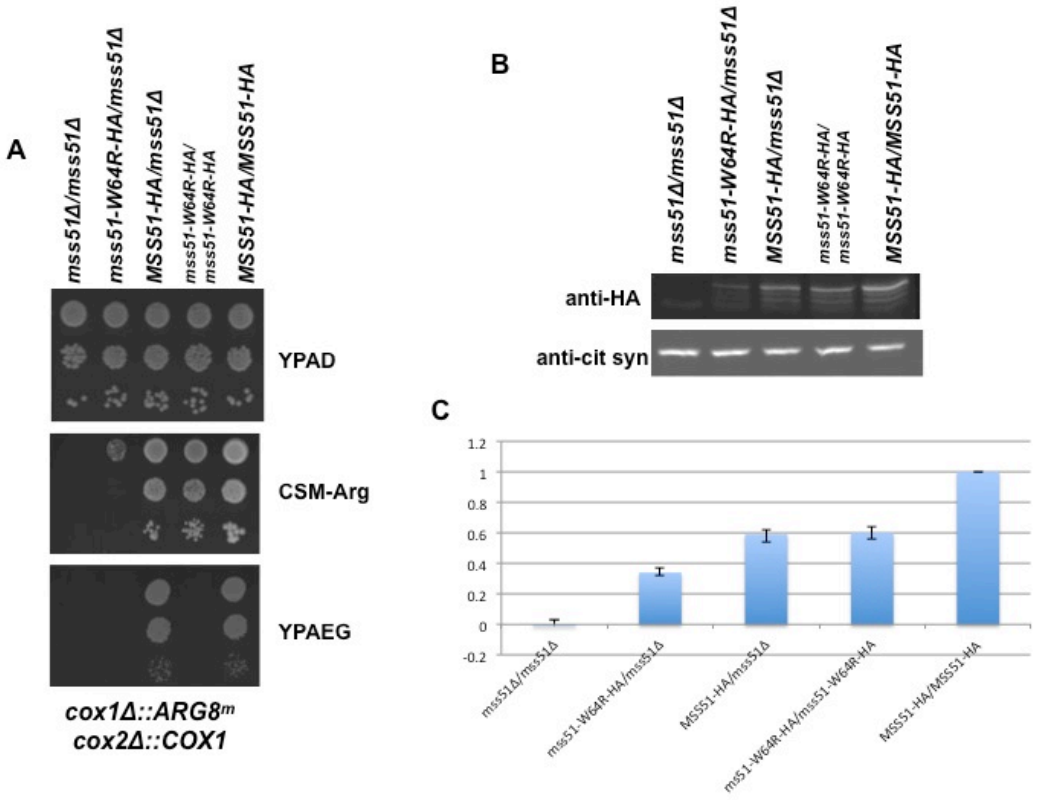
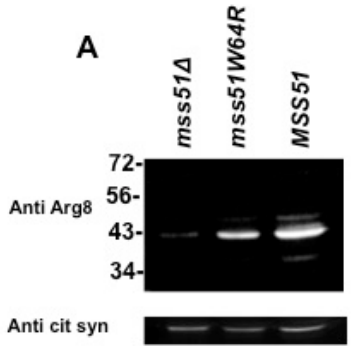


Figure 4.3 – The nonrespiratory phenotype of *mss51-W64R-HA* is not due to low levels of Mss51-W64R protein. Diploids with the indicated *MSS51* genotypes were created, each in a strain bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA. (A) Ten-fold serial dilution of cells were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. (B) Quantitative fluorescence Western blot analysis of total protein prepared from strains with the indicated relevant nuclear genotype, probed with anti-HA and anti citrate synthase antibodies. (C) In each strain, units anti-HA per units anti-citrate synthase was determined, and normalized to *MSS51::3xHA/MSS51::3xHA,* which was set to 1 within each experiment. The solid bar represents the average and error bars are the range (n=3). Strains were: *mss51Δ/ mss51Δ,* ZV68; *mss51-W64R-HA/mss51Δ*, ZV69; *mss51-W64R-HA/mss51-W64R-HA*, ZV56; *MSS51-HA/mss51Δ*, ZV70; *MSS51-HA/MSS51-HA,* ZV71. See Table 4.1 for complete genotypes.

mss51-W64R does not prevent read through of _COX1_ coding sequence into the downstream reporter, and can stabilize low levels of Cox1p

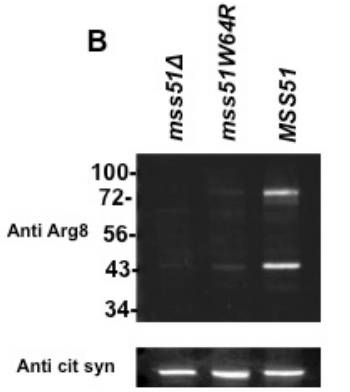
In order to determine the efficiency of *mss51-W64R* at activating translation at the *COX1* 5' UTR, I performed an anti-Arg8 Western analysis of *mss51-W64R* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1*mtDNA (Figure 4.4A). I found that steady state Arg8 levels are present at approximately 40% of WT, suggesting that *mss51-W64R* has decreased translation activation activity at the *COX1* 5' leader. As previously described, an anti-Arg8 Western of *MSS51* in a strain bearing *COX1(1-512)::ARG8^{m}* displayed two bands, a large band corresponding to Cox1-Arg8m and a smaller band corresponding to Arg8m (Perez-Martinez, Broadley et al. 2003). An anti-Arg8 fluorescence Western analysis of *mss51-W64R* in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA revealed the presence of two similar bands, which suggests that *mss51-W64R* can read through the complete *COX1* message and can produce steady state Cox1-Arg8 (Figure 4.4B). Steady state Cox1-Arg8m protein levels produced by *mss51-W64R* are reduced to approximately 1/15th of *MSS51* levels, consistent with the results obtained with *mss51-52* (Figure 3.2). However, taking into account the fact that *mss51-W64R* only produces Arg8p at the *COX1* leader 40% of WT, the addition of *COX1* to *ARG8^{m}* decreases the amount of the Cox1-Arg8p fusion protein 6 fold. I propose that the additional decrease in Cox1-Arg8 levels is likely due to a combination of Cox1p synthesis and stability defects of *mss51-W64R.* The ratio of Cox1-Arg8m to Arg8, between *mss51-W64R* and *MSS51* is similar suggesting that cleavage of Arg8m is not adversely affected in the mutant.



Averages (n=3) relative to citrate synthase:

cox1Δ::ARG8^m cox2Δ::COX1 mtDNA

	mss51-W64R	*MSS51*	*mss51-W64R/ MSS51*	STDEV
anti Arg8	0.07	0.16	0.427	0.077



Averages (n=3) relative to citrate synthase *COX1(1-512)::ARG8^m* mtDNA

	mss51-W64R	*MSS51*	*mss51-W64R/ MSS51*	STDEV
anti Arg8 lower band	0.09	1.20	0.075	0.024
anti Arg8 upper band	0.06	0.86	0.052	0.023
Ratio lower:upper	1.53	1.41		
STDEV	0.04	0.13		

Figure 4.4 – *mss51-W64R* is capable of promoting weak read through of *COX1* coding sequence into downstream reporter in a strain bearing *COX1(1-512)::ARG8^m* mtDNA. Strains containing (A) *cox1Δ::ARG8^m cox2Δ::COX1* and (B) *COX1(1-512)::ARG8^m* were grown to mid-log phase in liquid complete raffinose medium, and total proteins were prepared. 35 ug total protein was separated by 12.5% SDS-PAGE, transferred to Immobilon-FL membrane and probed with anti-Arg8 primary antibody, Alexofluor 488 goat anti-mouse secondary antibody, and visualized using a fluorescence detection imager. The membranes were stripped using a low pH buffer, and re-probed with an anti-citrate synthase primary antibody, Alexofluor 488 goat anti-mouse secondary antibody, and visualized using a fluorescence detection imager. Strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-W64R,* ZV48a; *MSS51,* XPM171. Strains bearing *COX1(1-512)::ARG8^m* mtDNA were: *mss51Δ*, XPM76; *mss51-W64R,* ZV50a; *MSS51,* XPM78. See Table 4.1 for complete genotypes.

To determine if *mss51-W64R*'s translation of *COX1* can be detected, mitochondrial translation products were [^{35}S] methionine radiolabeled for 20 minutes from strains bearing wildtype, *COX1(1-512)::ARG8m* or *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA. In a strain containing *mss51-W64R* and bearing wild type mtDNA, no *COX1* translation was detected (Figure 4.5A). A shorter 10 minute pulse and no chase did not result in detectable levels of *COX1* translation (data not included). In wild type mtDNA, I propose that *COX1* translation is occurring in strains containing *mss51-W64R*, but *COX1* is being degraded more rapidly than it can be labeled.

[^{35}S] methionine radiolabeled mitochondrial translation products in a strain containing *mss51-W64R* and bearing *COX1(1-512)::ARG8^{m}* mtDNA shows a small amount of *COX1* translation occurring (Figure 4.5B). *mss51-W64R* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA also shows low levels of *COX1* translation (Figure 4.5C).

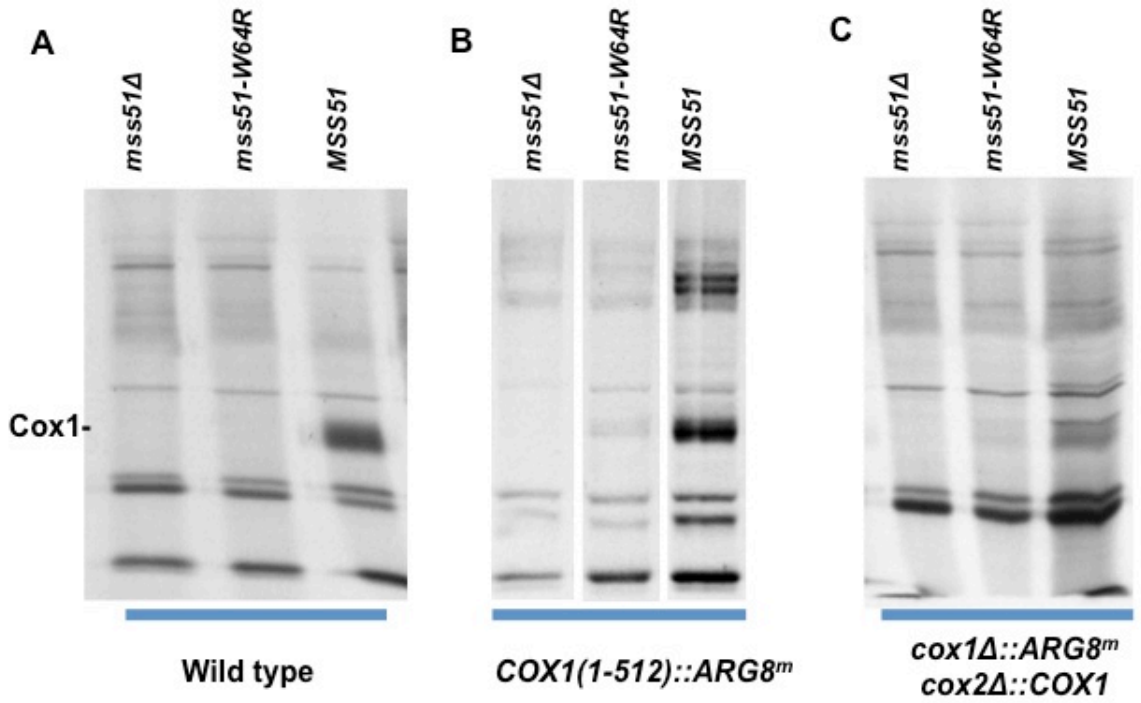


Figure 4.5 – *mss51-W64R* can translate low levels of *COX1* when either the C terminus of Cox1 is modified, or *COX1* translation activation is independent of *mss51-W64R.* Strains were grown to mid-log phase in liquid raffinose media. Cells were labeled for 20 minutes with [^{35}S] methionine in the presence of cycloheximide, and chased with cold methionine for 10 minutes. Crude mitochondria were prepared and separated using 12.5% SDS-PAGE, and visualized by autoradiography. These labelings have been repeated once. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-W64R*, ZV43; *MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8^{m}* mtDNA were: *mss51Δ*, XPM76; *mss51-W64R,* ZV50a; *MSS51,* XPM78. Strains bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-W64R,* ZV48a; *MSS51,* XPM171. See Table 4.1 for complete genotypes.

It is surprising that *COX1* translation can be observed in a strain with *mss51-W64R* and bearing *COX1(1-512)::ARG8^{m}* mtDNA but not in a strain bearing wild type mtDNA. I propose that *ARG8^{m}* alters or protects the C terminus the fusion protein, as it is known that the C terminal residues of *COX1* are involved in feedback regulation and could be the location at which Mss51 interacts with (Shingu-Vazquez, Camacho-Villasana et al. 2010). In their paper, Shingu-Vazquez et al. (2010) propose that the C terminus of Cox1 can alter its conformation due to interactions with assembly factors. It is possible that the Arg8 protein changes the Cox1 C terminal structure sufficiently to enhance translation and enable *mss51-W64R's* weak stabilization of the Cox1-Arg8^{m} protein. *COX1* translation can also be observed in strain bearing *COX1* flanked by *COX2* UTRs, which I explain by a quantitative effect of higher levels of *COX1* translation activation by *PET111*.

In order to determine if *mss51-W64R* allows for production of stable Cox1 protein, an anti-Cox1 Western was performed from crude mitochondria samples prepared from strains with *mss51-W64R* and bearing wild type, *COX1(1-512)::ARG8^{m},* or *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA (Figure 4.6). I found that *mss51-W64R* can produce highly reduced levels of steady state Cox1 relative to *MSS51* when either the C terminus of *COX1* is modified in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA, or in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, where *COX1* translation activation is independent of *mss51-W64R*.

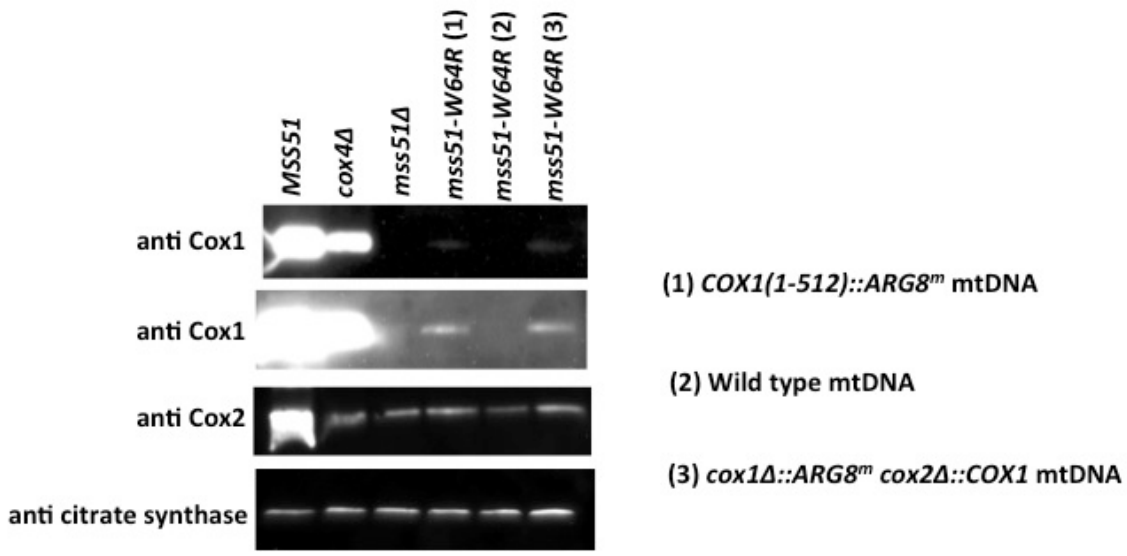


Figure 4.6 – *mss51-W64R* can produce low levels of steady state Cox1p when either the C terminus of Cox1 is modified, or *COX1* translation activation is independent of *mss51-W64R*. Strains were grown in complete raffinose medium and crude mitochondria were prepared. 25 ug mitochondria were subjected to 12.5% SDS-PAGE and transferred to a PVDF membrane, which was probed with an anti-*COX1* primary antibody (Invitrogen), an anti mouse – HRP secondary antibody, and visualized with ECL. This experiment has been repeated once. Strains were: *MSS51,* DAU1; *cox4Δ,* XPM51, *mss51Δ*, XPM89; *mss51-W64R COX1(1-512)::AR8*^{m} mtDNA, ZV50a; *mss51-W64R* wild type mtDNA, ZV43; *mss51-W64R cox1Δ::ARG8*^{m} *cox2Δ::COX1*mtDNA, ZV48. See Table 4.1 for complete genotypes.

Mss51-W64R is not sequestered in a Cox1 intermediate assembly complex

It has been observed that most Mss51 is found sequestered in a large 450 kDa complex, but in a nonrespiratory *cox14Δ* that does not accumulate stable Cox1p, Mss51 is found in to accumulate in a 120 kDa complex (Fontanesi, Soto et al. 2009). We asked if Mss51-W64R would accumulate in the larger or smaller molecular weight complex. If Mss51-W64R were to accumulate in the larger 450 kDa complex, but still be unable to support respiration, it would suggest that the nonrespiratory phenotype was due to a failure of Mss51-W64R to disengage with from the Cox1 assembly intermediate and reinitiate new translation at the *COX1* 5' leader. Conversely, accumulation of Mss51-W64R in the smaller complex, resembling a *cox14Δ* strain, would suggest that the inability of Mss51-W64R to support respiratory growth is due to its failure to stabilize Cox1. I found that Mss51-W64R accumulates in a low molecular weight complex that resembles a *cox14Δ* (Figure 4.7). As expected, Mss51 accumulates in a larger molecular weight complex in a *cox2Δ*, as downstream assembly of Cox1 is blocked. That the accumulation of Mss51-W64R most closely resembles a *cox14Δ* strain with wild type mtDNA suggests that *mss51-W64R* is unable to form the higher molecular weight intermediate Cox1 assembly complex, likely due it is inability to produce steady state Cox1p at levels approaching WT.

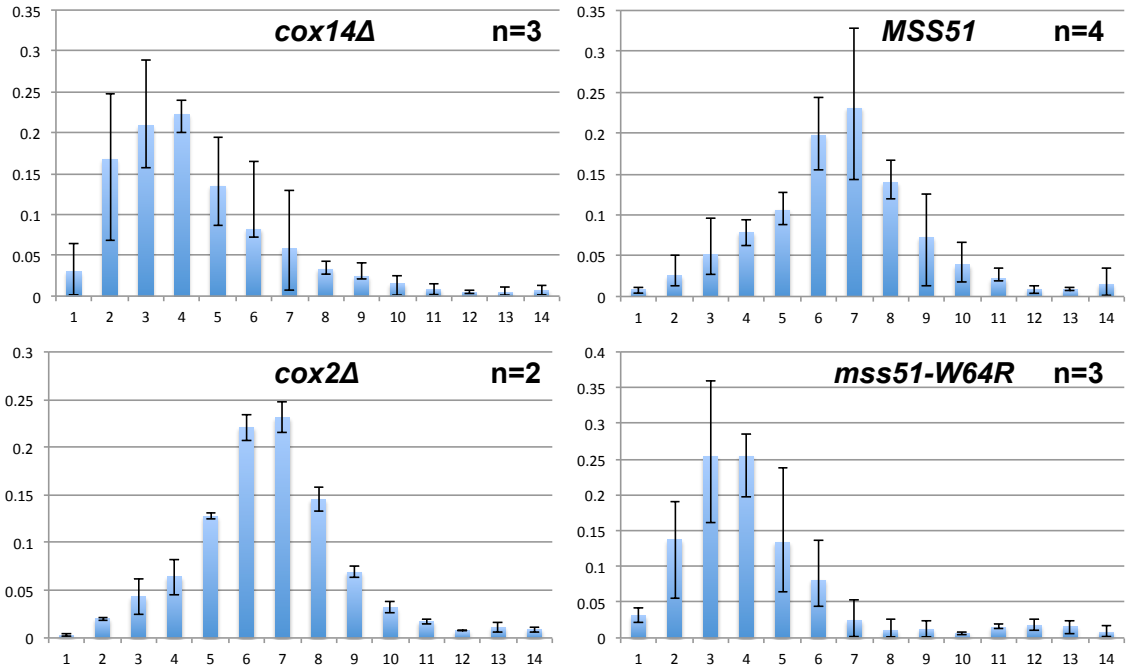


Figure 4.7 – Mss51-W64R accumulates in low molecular weight complexes, and resembles Mss51 sedimentation in a *cox14Δ* strain that fails to produce stable Cox1p. Strains were grown in raffinose medium, gradient purified mitochondria were prepared, solubilized with digitonin and centrifuged on a 7-20% sucrose gradient (see Methods and Materials). 14 equal aliquots were collected and 100 uL of each aliquot was Strataclean precipitated. Samples were separated on 12.5% SDS-PAGE and transferred to a Immobilon-FL membrane, which was probed with anti-*MSS51* primary antibody (Barrientos, Zambrano et al. 2004), Alexofluor 488 goat anti-rabbit fluorescence secondary antibody, and visualized using a fluorescence detection imager. The solid bar represents the average, error bars are the range, and n is the number of repeats. Strains were: *cox14Δ,* CAB267; *MSS51,* TF258; *cox2Δ*, TF245; *mss51-W64R*, ZV43. See Table 4.1 for complete genotypes.

An *oma1Δ* or *yme1Δ* does not enable *mss51-W64R* to stabilize *COX1* in a strain bearing wild type mtDNA

It has been previously reported that the deletion of the metalloendopeptidase *OMA1* was able to suppress the lack of respiration caused by a *coa2Δ* or a *shy1Δ,* but not a *coa1Δ* (Khalimonchuk, Jeong et al. 2012). We asked to see if deleting *OMA1* would restore *COX1* translation when combined with *mss51-W64R* in a strain bearing wild type mtDNA, and found that an *oma1Δ* was unable to restore translation in a strain with *mss51-W64R* and bearing wild type mtDNA (Figure 8). We also tested to see if deleting *YME1*, the catalytic subunit of the i-AAA mitochondrial protease could allow *mss51-W64R* to translate *COX1*, and found that similar to an *oma1Δ,* a *mss51-W64R yme1Δ* strain bearing wild type mtDNA was unable to translate *COX1* (Figure 4.8).

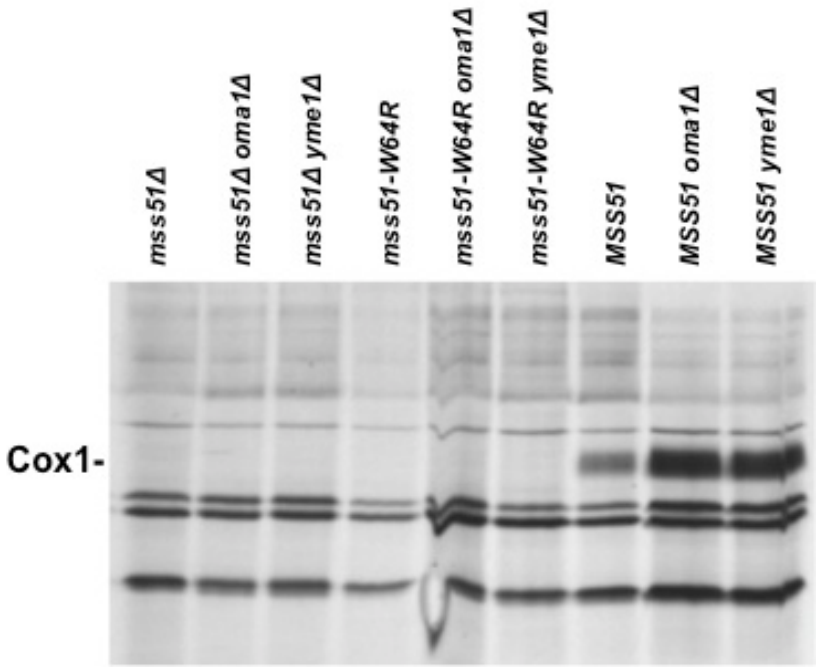


Figure 4.8 – An *oma1Δ* or *yme1Δ* does allow detection of *COX1* by [^{35}S] methionine pulse labeling of *mss51-W64R* in strains bearing wild type mtDNA. Strains were grown to mid-log phase in liquid raffinose complete medium. Cells were labeled for 10 minutes with [^{35}S] methionine in the presence of cycloheximide with no chase. Crude mitochondria were prepared and separated using 12.5% SDS-PAGE, and visualized using film. This labeling has not been repeated. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51Δ oma1Δ,* ZV72; *mss51Δ yme1Δ,* ZV73; *mss51-W64R*, ZV43; *mss51-W64R oma1Δ*, ZV74; *mss51-W64R yme1Δ*, ZV75; *MSS51,* DAU1; *MSS51 oma1Δ,* ZV76; *MSS51 yme1Δ,* CAB38. See Table 4.1 for complete genotypes.

An *oma1Δ* does not affect the phenotype of *mss51-W64R* in a strain bearing *COX1(1-512)::ARG8^m* mtDNA

I asked if an *oma1Δ* would affect either the arginine or respiratory phenotype of *mss51-W64R* in a strain bearing *COX1(1-512)::ARG8^m* mtDNA, and found no phenotypic effect of an *oma1Δ* on either *mss51Δ, mss51-W64R* or *MSS51* (Figure 4.9). It appears that the ability of an *oma1Δ* to suppress the nonrespiratory phenotype of *coa2Δ* or a *shy1Δ* does not extend to an interaction with *mss51-W64R.*

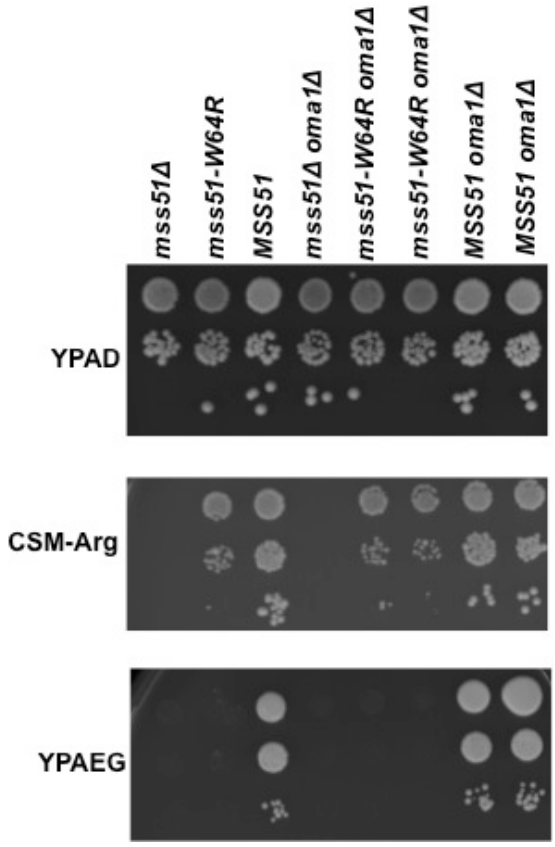


Figure 4.9 - An *oma1Δ* does not affect the phenotype of an strain containing *mss51-W64R* bearing *COX1(1-512)::ARG8*^{m} mtDNA. Ten-fold serial dilution of cells bearing *COX1(1-512)::ARG8*^{m} mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for or 3 days at 30ºC. This dilution series has been repeated once. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM89; *mss51-W64R*, ZV43; *MSS51,* DAU1. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM76; *mss51-W64R,* ZV50a; *MSS51,* XPM78, *mss51Δ oma1Δ*, ZV77; *mss51-W64R oma1Δ,* ZV78; *MSS51 oma1Δ,* ZV79. See Table 4.1 for complete genotypes.

DISCUSSION

The goal of this project to generate a separation-of-function *mss51* mutant, and to use the information gained from the *mss51* mutants to elucidate domains or motifs of *MSS51*. At present, *MSS51* has no motifs described in the SGD (yeastgenome.org) that are responsible for its functions. *mss51* dominant alleles F199I and T167R have previously been identified to suppress the nonrespiratory phenotype of *shy1* deletion in a W303 strain (Barrientos, Barros et al. 2002), although in a D273-10B strain a shy1 deletion does not show a respiratory defect.

I propose that *mss51-W64R* is indeed a separation of function mutant, as opposed to simply an allele with decreased global function, based on start codon mutations introduced into the *COX2* start codon when in a strain bearing *cox2(1-91)::ARG8m* mtDNA (Bonnefoy and Fox 2000). Bonnefoy and Fox found that when certain *COX2* start codon mutations were introduced, growth on media lacking arginine was abrogated prior to the loss of respiratory growth. If the same principles are applied to *COX1*, an *mss51* mutant with decreased function in a strain bearing *COX1(1-512)::ARG8*^{m} mtDNA background would be Arg- but able to support respiratory growth. As *mss51-W64R* has the converse phenotype in this mtDNA background, I feel that *mss51-W64R* is a separation of function mutant. The possibility remains that *COX1(1-512)::ARG8*^{m} behaves differently than *COX2(1-92)::ARG8*^{m}, and in order to test this possibility *COX1* start codon mutation analysis would need to be performed in a manner similar to Bonnefoy and Fox 2000.

When translationally fused to the C terminus of *COX2(1-91)*, production of Arg8p was proposed to accurately reflect *COX2* synthesis rates (Bonnefoy and Fox 2000). It is currently not known if *COX1::ARG8m* mtDNA behaves in a similar manner. When quantitative fluorescence Western analysis was performed in figure 4B, the ratio of Cox1-Arg8 to Arg8 in *mss51-W64* and *MSS51* were roughly equivalent. This suggests that *mss51-W64R* does not preferentially destabilize the Cox1 coding sequence, which could leave the Arg8m protein intact.

It is not entirely clear if there is a difference in the ability to promote read through of Cox1 between *mss51-W64R* and *mss51-52* in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA. As shown in Figure 2 of Chapter 3, *mss51-52* does not produce steady state Cox1-Arg8p at levels anywhere approaching *MSS51,* if there is any Cox1-Arg8p detectable at all. *mss51-W64R* has been demonstrated to produce some Cox1-Arg8p as demonstrated in Figure 4B of this chapter; however, the level of Cox1-Arg8p produced by *mss51-W64R* is vastly reduced relative to that produced by *MSS51.* While *mss51-W64R* is more efficient at promoting growth on medium lacking arginine than *mss51-52* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* (Figure 3.8) the ability of *mss51-W64R* to promote full read through of the *COX1* gene into the downstream reporter in a strain bearing *COX1(1-512)::ARG8^{m}* may not be significantly increased.

In addition to *mss51-W64R*, I removed the missense W64R mutation from *mss51-52*, creating the allele *mss51-54. mss51-54* was able to support near WT levels of respiratory growth in strains bearing either wild type, *COX1(1-512)::ARG8^{m}*, or

cox1Δ::ARG8^m cox2Δ::COX1 mtDNA, as shown in Figure 8 of Chapter 3. *mss51-54* does not appear to exhibit a mutant phenotype, so its 5 missense mutations likely do not affect its abilities of translation activation and Cox1 synthesis and assembly.

mss51-W64R represents the first separation-of-function *mss51* mutant. Information gleaned from *mss51-W64R* supports and builds upon the existing model for *MSS51* function. *mss51-W64R* reinforces the claim that *MSS51* has three distinct and separable functions – *COX1* translation activation through the 5' UTR, read through of *COX1* message, and assembly of Cox1 into cytochrome *c* oxidase. *mss51-W64R* can activate translation at the *COX1* 5' UTR, as demonstrated by the Arg(+) phenotype in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1*mtDNA. *mss51-W64R* weakly promotes read through the *COX1* message, as evidenced by the Cox1-Arg8m band in an Arg8 Western blot analysis in a strain bearing *COX1(1-512)::ARG8^m* mtDNA. *mss51-W64R* is more efficient operating through the *COX1* leader and activating translation of downstream reporter than forming the early assembly complex. I therefore propose that the single missense mutation of *mss51-W64R* renders it selectively deficient at a process downstream of *COX1* translation activation, but I have been unable to conclusively demonstrate what step this may be.

Concerning the suppression of *mss51-52* by the Trp tRNA tW(CCA)K with the A -> G mutated anticodon (strain ZV80), it would be interesting to determine how often tryptophan is inserted at amino acid 64 instead of arginine by the informational suppressor. The *mss51-52* allele is tagged with a triple HA epitope on

the C-terminus, and by pulling down *mss51-52* with anti-HA beads and submitting the protein for analysis by mass spectrometry, it could be possible to estimate the suppression efficiency of the mutated tW(CCA)K tRNA by comparing the ratio of mss51-W64 : mss51-R64.

Table 4.1 – *S. cerevisiae* strains used in Chapter 4		
Strain	**Genotype**	**Reference**
CAB267	MATα, *ade2, ura3-Δ, MSS51::3xHA, cox14Δ::URA3* [ρ+]	This study
CAB38	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, his3Δ-HindIII, yme1Δ::URA3* [ρ+]	This study
DAU1	MATa, *ade2, ura3-Δ* [ρ+]	(Costanzo and Fox, 1988)
TF245	MATa, *lys2* [ρ+, *cox2-62*]	This study
TF258	MATα, *lys2, leu2-3,112, ura3-55, his4-519, MSS51::3xHA, COX14::3xMYC* [ρ+]	This study
XPM51	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *cox4Δ::LEU2*]	This study
XPM76	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM78	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM89	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+]	This study
XPM171	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
XPM174	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
ZV43	MATa, *lys2, leu2-3,112, arg8::hisG, ura3::kanMX3, mss51-W64R::3xHA, COX14::3xMYC* [ρ+]	This study
ZV48a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC* [ρ+, *cox1Δ::ARG8m,*	This study

	cox2Δ::COX1c, COX2]	
ZV56	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3::kanMX3/ura3::kanMX3, mss51-W64R::3xHA/mss51-W64R::3xHA, COX14::3xMYC/COX14::3xMYC* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV68	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, his3Δ-HindIII/HIS3, mss51Δ::LEU2/mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV69	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3::kanMX3/ura3-52, mss51-W64R::3xHA/mss51Δ::LEU2, COX14::3xMYC/COX14* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV70	MATa/MATα, *lys2/lys2, leu2-3,112/LEU2, arg8::hisG/ARG8, ura3/ura3-52, MSS51::3xHA/mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV71	MATa/MATα, *lys2/LYS2, arg8::hisG/ARG8, ura3-52/ura3Δ, ade2/ADE2, MSS51::3xHA/MSS51::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV72	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2, oma1Δ::kanMX3* [ρ+]	This study
ZV73	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2, yme1Δ::URA3* [ρ+]	This study
ZV74	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC, oma1Δ::kanMX3* [ρ+]	This study
ZV75	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC, yme1Δ::URA3* [ρ+]	This study
ZV76	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, oma1Δ::kanMX3* [ρ+]	This study
ZV77	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2, oma1Δ::kanMX3* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV78	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-W64R::3xHA, COX14::3xMYC, oma1Δ::kanMX3* [ρ+,	This study

	COX1(1-512)::ARG8m, ΔΣaI, ΔΣbI]	
ZV79	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, oma1Δ::kanMX3* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study

All strains are congenic or isogenic to D273-10B, except ZV80 and NAB69 rho0

Mitochondrial genotypes are shown in brackets. ΔΣaI, ΔΣb refers to an intronless mitochondria derived from CK520

c Ectopic insertion of the chimeric COX1 upstream of COX2

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Chapter 5

mss51-102 strongly supports respiration when the *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence

INTRODUCTION

mss51-102 and *-103* support a strong Arg+ and very weak Pet+ phenotype in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA. However, *-102* and *-103* respire at near WT levels in strains bearing either *COX1(1-512)::ARG8^m* or wild type mtDNA. In this chapter, I investigate the cause of the slow respiratory ability of *mss51-102* and *mss51-103*, and I propose an undescribed function of *MSS51* based on the behavior of *mss51-102* and *mss51-103.*

METHODS AND MATERIALS

Strains, media and genetic methods

Saccharyomyces cerevisiae strains used in this chapter are listed in Table 5.5. See Chapter 2 for media and genetic methods.

Purified mitochondrial DNA preparation, Southern blot analysis

Preparation and isolation of highly purified mitochondria were carried as previously described (Folley and Fox 1991). Southern blots were performed as previous described (Brown 2001). Primers CO1F6-5' (5' ATG GTA CAA AGA TGA TTA TAT TCA ACA AAT GC 3') and Fusion 4R (5' TTA TTG TTT AAT CCA TTA AC 3') were used on DNA from CK520 to create the *COX1* probe. Primers COX2EHW38 (5' GCA TGT TAT TTT CAG GAT TCA G 3') and 127AMB (5' CTG AAT ATT CAT ACT ATC AAT ATC ATT GAT ATC C 3') were used on DNA from CK520 to create the *COX2* probe. Primers SS-8 (5' GAT AAA GCA CCC ATT GTT CTA CCA 3') and ARG8mEHW4 (5' ATG TTC AAA AGA TAT TTA TC 3') were used on DNA from ZV27a to create the *ARG8^m* probe.

PCRs to analyze ZV65 mitochondrial DNA rearrangement

The PCR of *COX1* 5' UTR forward, *COX1* coding sequence reverse used primers Cox15'UTRF3 (5' GAA TTG TTT AAA GTT ATA AC 3') and Cox1CodSeqR1 (5' CTA ATG ATT AAA GAC ATT GCT G 3'). The PCR of *COX1* 5' UTR forward, *ARG8^m* coding sequence reverse used primers Cox15'UTRF3 (5' GAA TTG TTT AAA GTT ATA AC 3') and Arg8 Cod Seq R3 (5' CAT TGA ATT TCA TCA TGA ATG 3'). The PCR of *COX2* 5'

UTR forward, *COX1* coding sequence reverse used primers Cox25'UTR F2 (5' GGA GGA CCG AAG GAG TTT TAG 3') and COX1LSB8 (5' TCT ACA CTA GGT CCT GAA TGT GC 3'). The PCR of *COX2* 5' UTR forward, *ARG8^m* coding sequence reverse used primers xpcox2F4 (5' ATA ATA AAG ATA TCA TAA ATT TTT AAT AAA AGT AG 3') and Fusion R2 (5' ATG AGC CCA TAG TTT ACC TG 3'). The PCR of *COX1* coding sequence forward, *COX1* 3' UTR reverse used primers CO1F7-3' (5' CTT GTA AAG GTC TAC CTA TCG GG 3') and CO1R10-3' (5' AGG ATT ACA TAT GTA TTT ATT TAT ATA GTT CCC CAA AAG G 3'). The PCR of *COX1* coding sequence forward, *COX2* 3' UTR reverse used primers Fusion 3F (5' TGG TGG TTC AAT TAG ATT AG 3') and XPCox2R9 (5' TAT TTA TAT TTA CGT AAT AAG GTG ATT GAA TAG 3'). The PCR of *ARG8^m* coding sequence forward, *COX1* 3' UTR reverse used primers Fusion 6F (5' AGT GAG GCT CAT CCG GAT AT 3') and CO1R10-3' (5' AGG ATT ACA TAT GTA TTT ATT TAT ATA GTT CCC CAA AAG G 3'). The PCR of *ARG8^m* coding sequence forward, *COX2* 3' UTR reverse used primers SS-12 (5' AAT CCA AGC TAA ATA TCC TAA TCA 3') and xpcox2R3 (5' TTT ATA TTG ATA TCA TAA GGT GAT TGA ATA G 3').

Ligation of mtDNA fragments into pBluescript KS+

E. coli containing pBluescript II KS+ plasmid was grown in LB + Amp and the plasmid was isolated using the Plasmid Miniprep Kit (QIAGEN). The vector was digested with BspDI (Invitrogen) and treated with CIAP (Invitrogen) to prevent self ligation. Bands containing *COX1* and *COX2* were isolated from an EtBr stained gel of 1 ug ZV65 mtDNA digested with MspI using the Gel Extraction Kit (QIAGEN). Ligations of the *COX1* and *COX2* band were separately set up with a vector:insert

ratio of 1:1, with a DNA concentration of 100 ng / uL. Ligations were heat shock transformed into DH5a cells (Invitrogen) and transformants were selected on LB + Amp plates.

Testing for mitochondrial stability

Strain ZV65 (*mss51-102*, ZV65 mtDNA) was streaked from a freezer stock to YPAD, then a large clump of cells were restreaked to YPAD. Each YPAD plate was replica plated to CSM-ARG and YPAEG in parallel, and also mated to a lawn of NAB75 (*MSS51, arg8Δ,* rho0). The mated plates were printed to CSM-ARG and YPAEG in parallel.

Western Blot analysis

Total protein preparation, crude mitochondrial preparation and Western blot analyses were carried out as described in Chapter 3.

***In vivo* labeling of cycloheximide treated cells**

[^{35}S] methionine labeling of mitochondrial proteins was carried out as described in Chapter 4.

Plasmid construction

Plasmid pZV23 contains *mss51-102::3xHA* digested from mss51-102p with HindIII and XbaI, and cloned into vector Yep352 using HindIII and XbaI. Plasmid pZV24 contains *COX1* fragment digested from ZV65 purified mtDNA using MspI, cloned into pBluescript II KS+ vector using BspDI (NEB). Plasmid pZV25 contains *COX2*

fragment digested from ZV65 purified mtDNA using MspI, cloned into pBluescript II KS+ vector using BspDI (NEB).

COX2 sequencing

To sequence D273 COX2 5' UTR, DAU1 gDNA was PCR amplified and the product was sequenced using primers tMF4 (5' GGT TCA ATT CCT TCT ACA AG 3') and COX2R2 (5' CTG AAT CCT GAA AAT AAC ATG C 3'). To sequence the *COX2* 3' UTR from pZV25, primers COX2EHW2 (5' TTG TGT GGG ACA GGT CAT GC 3') and Ble-21 (5' CCT CGA GGT CGA CGG TAT CG 3') were used. The *COX2* 3' UTR was not sequenced from DAU1 gDNA.

RESULTS

mss51-102 and *mss51-103* are Arg+ and very weakly Pet+ in strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA

mss51-102 and *msss51-103* in strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, are Arg+ Pet- after 3 day incubation (Figure 5.1). The Arg+ phenotype when *ARG8^m* is flanked by the *COX1* UTR's suggests that *-102* and *-103* are able to activate translation at the *COX1* 5' UTR, and the inability to support respiration suggests that these alleles are deficient in one or more steps involving the synthesis and assembly of Cox1p. Both mutants grow weakly on nonfermentable medium after ~8 days (Figure 5.1). *mss51-102* contains two amino acid substitutions, T295A and G427D, and *mss51-103* contains S51Y and S217P (Figure 5.2 and Table 5.1).

Table 5.1 – Silent and missense mutations in *mss51-102* and *mss51-103*

mss51-102		*mss51-103*	
bp	aa	bp	aa
A883G	T295A	T33C	silent
A1122G	silent	C152A	S51Y
G1280A	G427D	T291C	silent
		T649C	S217P
		A1191G	silent

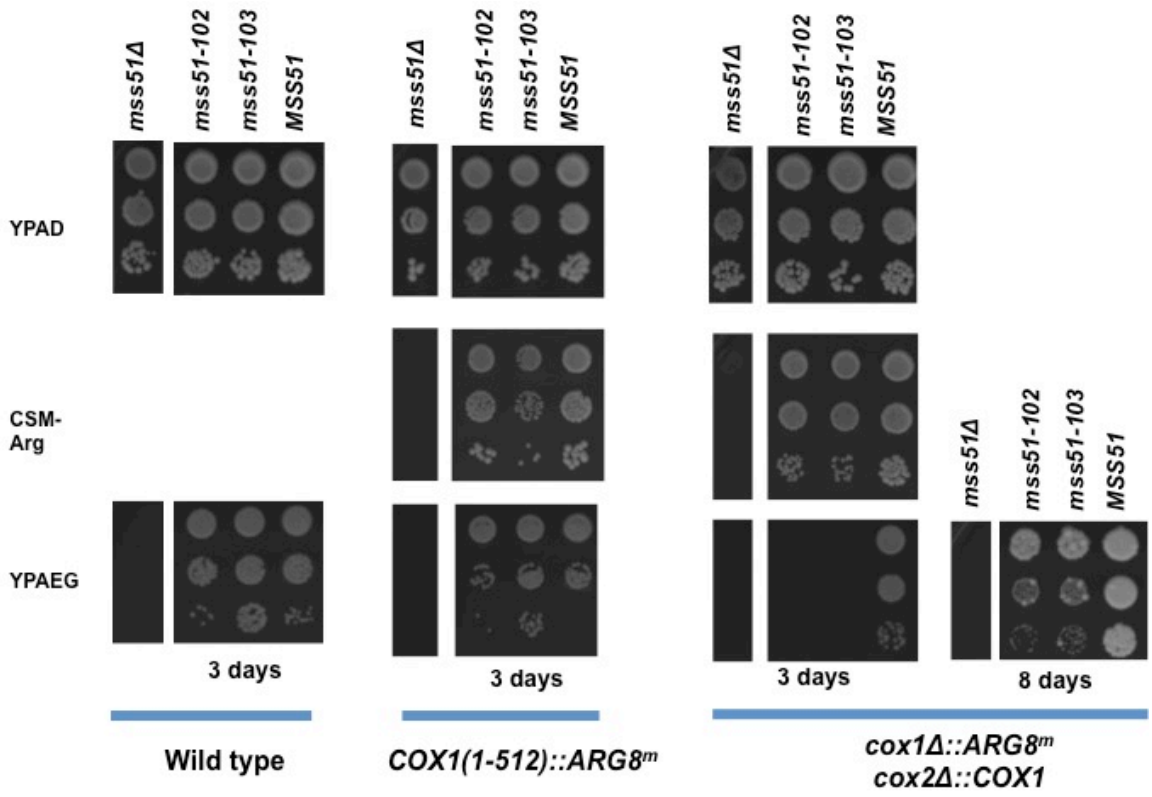

Figure 5.1 – Integrated *mss51-102* and *mss51-103* weak support respiration in strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1*mtDNA. Ten-fold serial dilution of cells bearing wild type, *COX1(1-512)::ARG8*^{m} or *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 or 8 days at 30ºC as indicated. Strains bearing wild type mtDNA were: *mss51Δ*, XPM89; *mss51-102*, CAB324; *mss51-103,* CAB325*; MSS51,* DAU1. This dilution series has been repeated once. Strains bearing *COX1(1-512)::ARG8*^{m} mtDNA were: *mss51Δ*, XPM76; *mss51-102*, ZV18; *mss51-103,* ZV25*; MSS51,* XPM78. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1*mtDNA were: *mss51Δ*, XPM174; *mss51-102*, ZV27; *mss51-103,* ZV22*; MSS51,* XPM171. See Table 5.5 for complete genotypes.

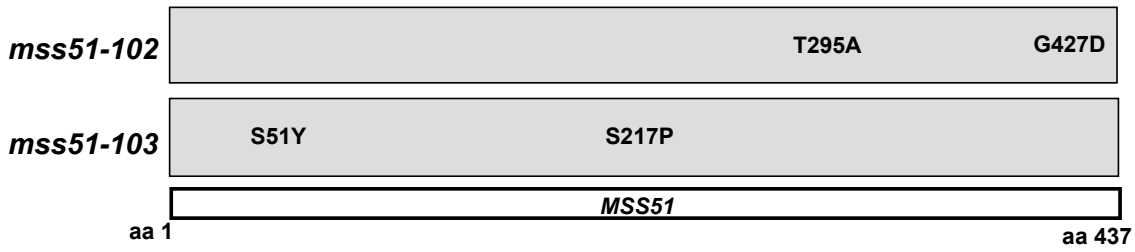

Figure 5.2 –A visual representation of *mss51* alleles *mss51-102* and *mss51-103*.

Silent mutations are not indicated.

mss51-102 and *mss51-103* support Arg+ and Pet+ growth of a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA, and also support Pet+ growth in a strain bearing wild type mtDNA (Figure 5.1). That *mss51-102* and *-103* are able to support respiration at near WT levels in strains bearing either *COX1(1-512)::ARG8^{m}* or wild type mtDNA suggests that there is a synthetic defect with *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA that causes respiration to be dramatically decreased.

mss51-102p is present at near Mss51p levels, and promotes full read through of the *COX1* message

mss51-102-HA has steady state protein levels reduced by approximately 25% relative to *MSS51-HA* in a strain bearing wild type mtDNA, as determined by the average of three anti-HA quantitative fluorescence Western blots (Figure 5.3). This small reduction steady state Mss51-102 protein is unlikely to be the cause of the inability of *mss51-102* to support respiration in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA.

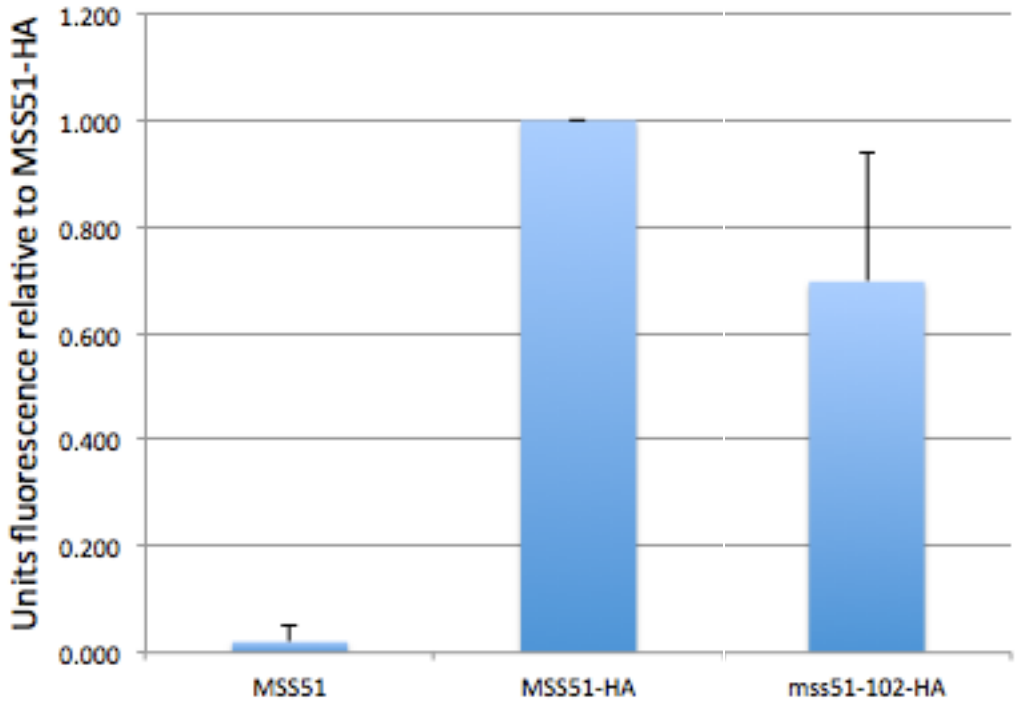

Figure 5.3 – *mss51-102* causes *mss51-HA* protein levels to be reduced approximately 25%. 35 ug of total proteins was separated by 12.5% SDS-PAGE. Quantitative fluorescence Western blot analysis of total protein prepared from strains with the indicated relevant nuclear genotype was performed using anti-HA and anti citrate synthase antibodies. In each strain, units anti-HA per units anti-citrate synthase was determined, and normalized to *MSS51::3xHA/MSS51::3xHA,* which was set to 1 within each experiment. The solid bar represents the average and error bars are the range (N=3). Strains bearing wild type mtDNA were: *MSS51,* DAU1; *MSS51::3xHA,* SB7; *mss51-102::3xHA,* ZV27a. See Table 5.5 for complete genotypes.

I predicted that *mss51-102* and *mss51-103* would be able to promote full read through of the *COX1* message and into the *ARG8^{m}* reporter in a strain bearing *COX1(1-512)::ARG8^{m}* mtDNA, based on their Arg+ and Pet+ phenotype in such a strain. When I performed an anti-Arg8 Western blot, and the larger Cox1-Arg8p fusion protein was detected in strains containing *mss51-102* or *mss51-103* and bearing *COX1(1-512)::ARG8^{m}* mtDNA (Figure 5.4). The presence of this band suggests that *-102* and *-103* are capable of promoting full read through of the *COX1* message into the downstream reporter.

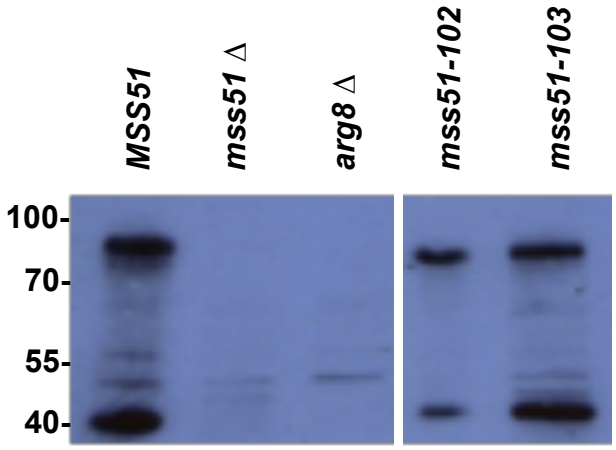

Figure 5.4 – *COX1(1-512)::ARG8*^{m} mtDNA provides a reporter for *COX1* translation. Relevant nuclear genotypes are as indicated. A 35 ug aliquot of a crude mitochondria preparation was separated by 12.5% SDS-PAGE, the Western blot was probed with anti-Arg8p antibody and visualized using ECL. This experiment has not been repeated. Strains were: *MSS51,* XPM78; *mss51Δ,* XPM76; *arg8Δ,* NB40-33A; *mss51-102*, ZV18; *mss51-103,* ZV25. See Table 5.5 for complete genotypes.

mss51-102 displays low levels of *COX1* translation in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA.

mss51-102 in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA promotes low levels of *COX1* translation relative to *MSS51* (Figure 5.5), which appears to correlate with the slow respiratory growth phenotype. The ability of *mss51-102* to activate translation at the *COX1* 5' UTR of the *ARG8^m* reporter is also decreased relative to *MSS51* in strains bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA (Figure 5.1). Taken together, these results indicate that *mss51-102* is capable of activating translation at the *COX1* leader, promoting full read through of the *COX1* message and translating *COX1*, but these activities are decreased relative to WT when the *COX1* 5' UTR is not present in *cis* to the *COX1* coding sequence.

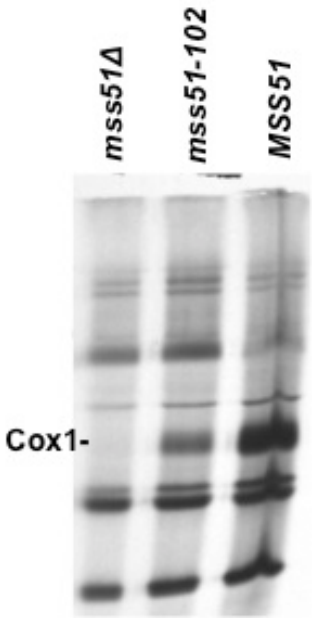

Figure 5.5 – [^{35}S] methionine labeling of mitochondrial translation products shows *mss51-102* translates low levels of *COX1* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA. Strains were grown to mid-log phase in liquid raffinose media. Cells were labeled for 20 minutes with [^{35}S] methionine in the presence of cyclohexamide, and chased with cold methionine for 10 minutes. Crude mitochondria were prepared and separated using 12.5% SDS-PAGE, and visualized using film. This labeling has not been repeated. Strains were: *mss51Δ*, XPM174; *mss51-102,* ZV27a; *MSS51,* XPM171. See Table 5.5 for complete genotypes.

Spontaneous enhanced respirers of *mss51-102* in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA can be identified

I asked to see if any factors could be identified which when overexpressed allowed *mss51-102* to exhibit enhanced respiration in strain ZV27a (*mss51-102, cox1Δ::ARG8^m cox2Δ::COX1* mtDNA). I utilized the yeast overexpression library from the Prelich lab (Jones, Stalker et al. 2008) (Open Biosystems). Approximately 11 fold coverage of the library was achieved and I identified 11 colonies that exhibited enhanced respiration linked to the plasmid. All candidate plasmids were found to contain *MSS51* (data not shown). I have been as yet unable to determine a gene other than *MSS51* which when overexpressed allows for enhanced respiration of *mss51-102* in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA.

In order to identify spontaneous enhanced respiring pseudorevertants of *mss51-102* weak respiratory phenotype in a strain bearing *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, 15 independent lawns of saturated YPAD cultures of ZV27a were plated to YPAEG media and screened for colonies that exhibited enhanced respiration after 3 days at 30ºC. 6 independent enhanced respiring pseudorevertants were identified, ZV60 – ZV65 (Figure 5.6). All of the pseudorevertants contained mutations that behaved in a dominant fashion; when crossed with XPM174 (*mss51Δ::LEU2*, *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA) the resulting diploids were Pet (+). When the enhanced respirers were made rho0 by EtBr treatment and crossed to XPM174, only 5 of the strains conferred enhanced respiration as a diploid, indicating that enhanced respiration of these 5 strains was

due to dominant nuclear mutations (Figure 5.7). A diploid of ZV65 rho0 x XPM174 respires in a similar manner to a diploid of ZV27a rho0 x XPM174, indicating that mutation conferring the enhanced respiration to ZV65 is mitochondrial (Figure 5.7).

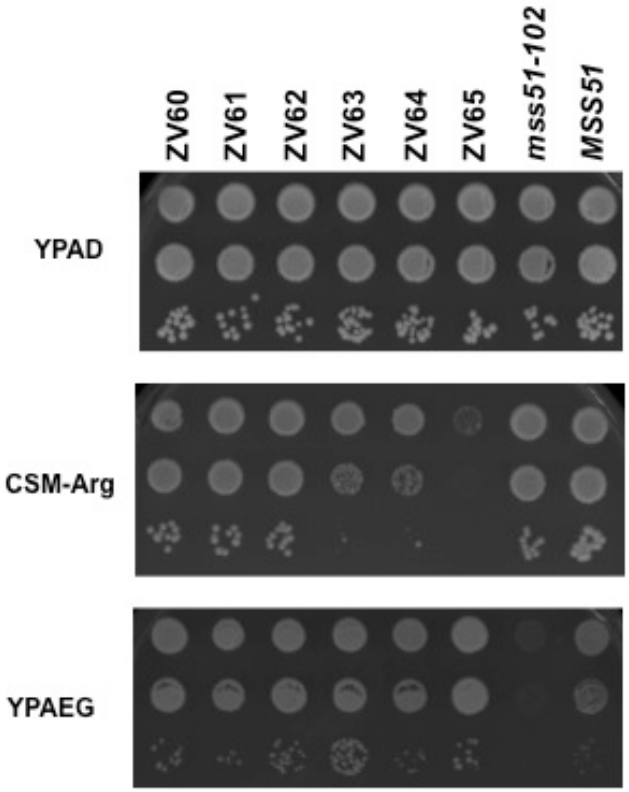

Figure 5.6 – 6 independent enhanced respirers of ZV27a were identified. Ten-fold serial dilution of cells were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. Strains were: *mss51-102,* ZV27a; *MSS51,* XPM171. This dilution series has been repeated once. See Table 5.5 for complete genotypes.

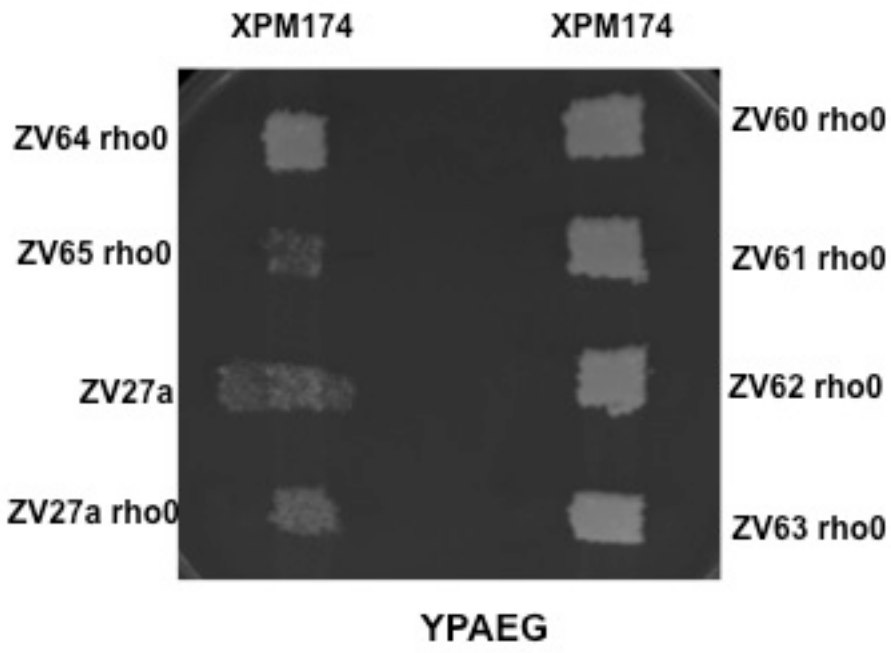

Figure 5.7 – Five enhanced respirers of ZV27a are nuclear dominant, and one enhanced respire of ZV27a is mitochondrial. Haploid cells of the indicated strains were patched in stripes. The stripes were cross-printed on YPAD medium and allowed to mate. The plate was then printed to complete ethanol/glycerol (YPAEG) medium. This cross has been repeated once. Plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

Three enhanced respirers of ZV27a contain mutations unlinked to *MSS51*

Three of the enhanced respiring strains, ZV60, ZV61 and ZV62, each contain a single dominant suppressor mutation unlinked to *MSS51* (Figure 5.8), which was found by dissecting tetrads of the enhanced respirers crossed to XPM174. In Figure 5.8, the *mss51-102* spores are Leu-, as XPM174 contains *mss51Δ::LEU2* and ZV27a has a *leu2-3,112* nuclear mutation. By looking at the respiratory phenotype of the Leu- spores, it was found that close to 50% of the Leu- spores were able to respire (Pet+), indicating that respiration is unlinked to *MSS51.* The numbers shown at the bottom are the total counts from several plates, and Figure 5.8 contains a representative plate of each cross.

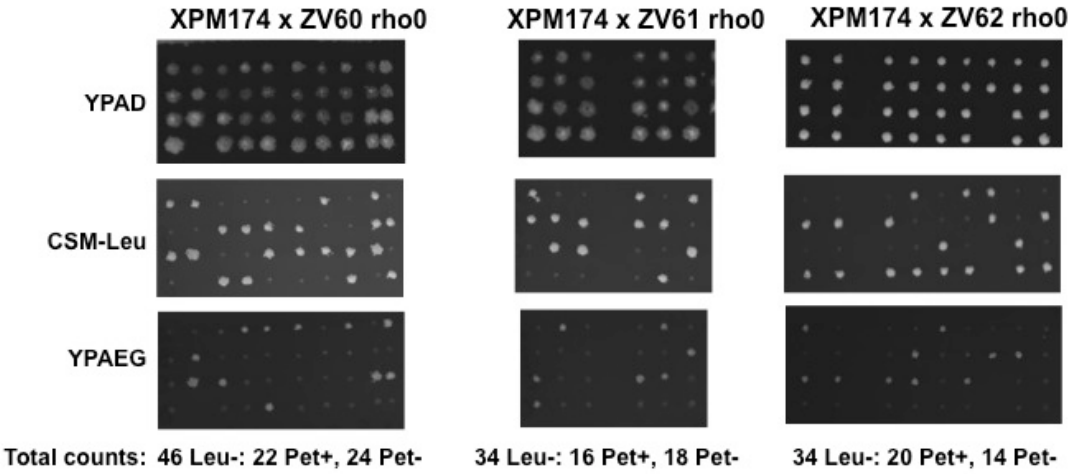

Figure 5.8 - 3 enhanced respirers of ZV27a have a single suppressor mutation unlinked to *mss51-102*. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on YPAD medium and allowed to mate. Diploids were selected and printed to sporulation media. Tetrads were dissected on complete medium (YPAD), and printed to medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG). All plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

I asked if these mutations could be informational suppressors, which I investigated by crossing ZV60, ZV61 and ZV62 to PTH60. PTH60 contains the *lys2-187* allele, discussed in Chapter 2. To my surprise, I found that the parent strain ZV27a, along with ZV60, ZV61 and ZV62 were all able to suppress the lys2-187 allele (Figure 5.9). As lys growth is 2:2 in almost all sets of tetrads, it exhibits non-Mendelian inheritance. Assuming the suppressor and *lys2-187* allele were unlinked, and the suppressor was informational, I would have expected lys growth would have gone (2+:2-),(1+:3-),(0+:4-) in a frequency of (1/6):(2/3):(1/6). It is possible that there exists a cytoplasmic [PSI] element in the parent ZV27a strain (Wickner, Masison et al. 1995). As a result of the ability of ZV27a to at on the lys2-187 allele, I am unable to conclude if ZV60, ZV61 and ZV62 contain informational suppressors. I have not identified the mutations in ZV60, ZV61 or ZV62 that allow for enhanced respiration.

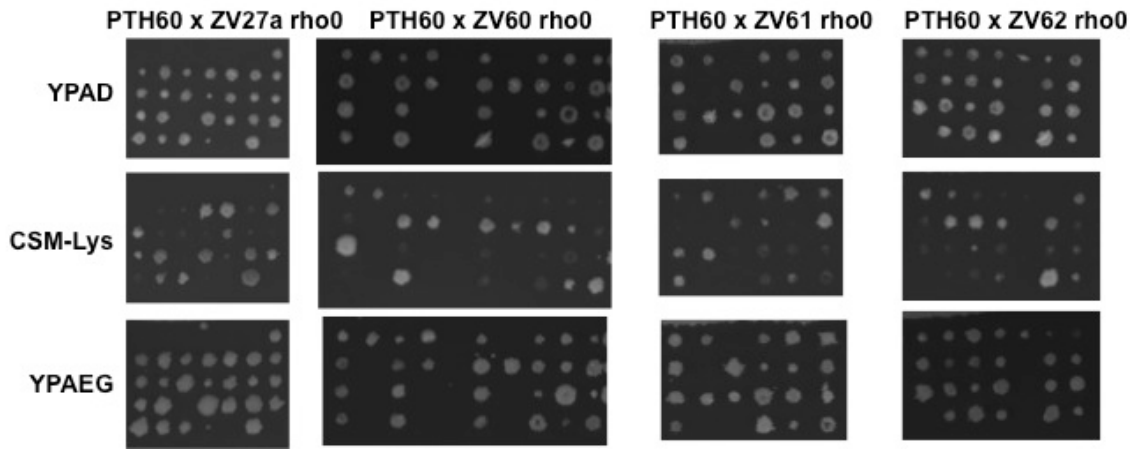

Figure 5.9 – ZV27a is able to suppress the *lys2-187* allele. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Diploids were selected and printed to sporulation media. Tetrads were dissected on complete medium (YPAD), and printed to medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG). All plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

Two enhanced respirers of ZV27a contain additional mutations in *mss51-102*

Two of the suppressors, ZV63 and ZV64, were found to be 100% linked to *mss51-102* (Figure 5.10). ZV63 rho0 and ZV64 rho0 were mated to XPM174, and by analyzing the *mss51-102* containing Leu- spores, it was found that every Leu- spore was able to respire, indicating that the respiratory phenotype was linked to *MSS51.* The numbers shown at the bottom are the total counts from several plates, Figure 5.10 contains a representative plate of each cross.

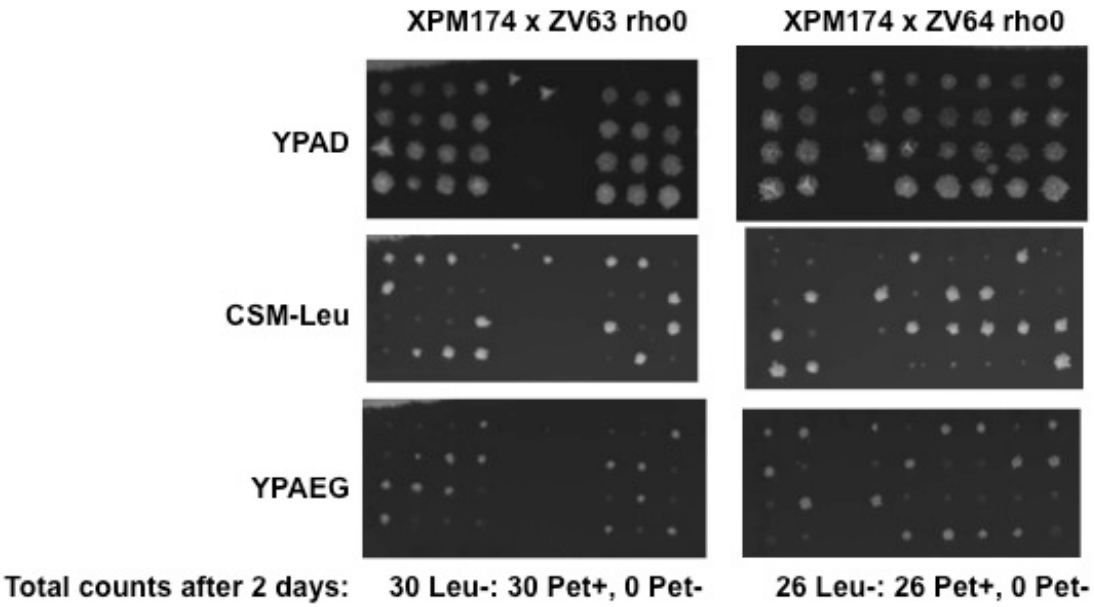

Figure 5.10 - 2 enhanced respirers of ZV27a contain a mutation linked to *mss51-102.* Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Diploids were selected and printed to sporulation media. Tetrads were dissected on complete medium (YPAD), and printed to medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG). All plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

I sequenced *mss51* and identified two separate second-site mutations. Either A219D in ZV63 (referred to as *mss51* allele *mss51-104)* or N205D in ZV64 (referred to as *mss51* allele *mss51-105)* were found to be able to enhance the respiratory phenotype of *mss51-102* in a strain bearing *cox1Δ::ARG8^{m} cox2Δ::COX1*. These alleles *mss51-104* and *-105* are not currently useful in determining any biologically relevant information concerning *MSS51*, but might become so if the crystal structure of *MSS51* is ever determined. A comparison of these additional mutations with the other *mss51* alleles is found in Table 5.2 and Figure 5.11.

Table 5.2 - Silent and missense mutations in *mss51* alleles, and changes in *MSS51* from S288C to D273 strain backgrounds

mss51-102		*mss51-103*		*mss51-104*		*mss51-105*		S288C to D273	
bp	aa	bp	aa	bp	aa	bp	aa	bp	aa
A883G	T295A	T33C	silent	C656A	A219D	A613G	N205D	G114A	silent
A1122G	silent	C152A	S51Y	A883G	T295A	A883G	T295A	G426A	silent
G1280A	G427D	T291C	silent	A1122G	silent	A1122G	silent	A507C	silent
		T649C	S217P	G1280A	G427D	G1280A	G427D	C816T	silent
		A1191G	silent					C928T	R310C
								C996G	silent
								C1124T	T375M
								G1190A	R397K

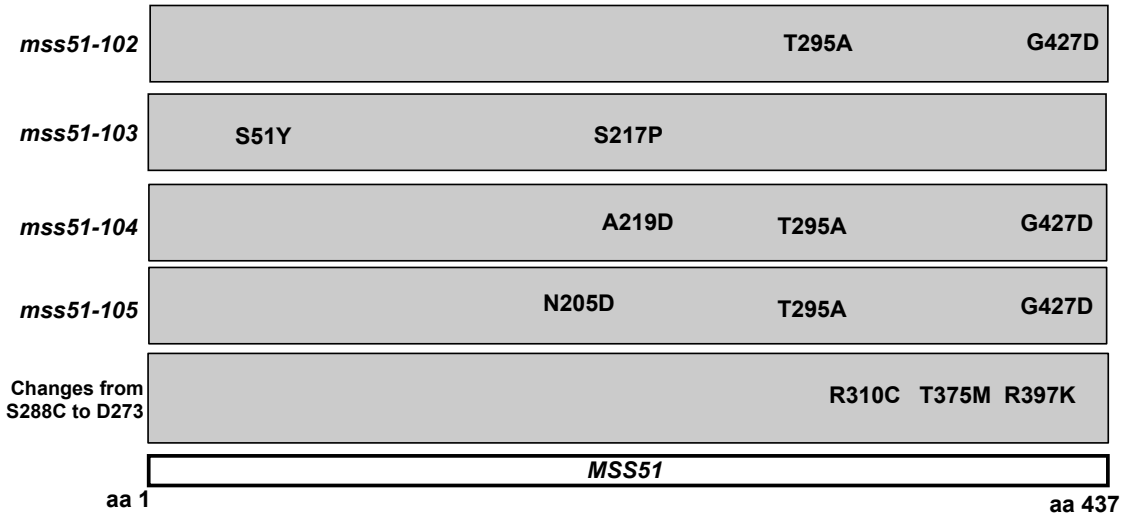

Figure 5.11 – A visual representation of *mss51* alleles *mss51-104* and *mss51-105*, and missense mutations of *MSS51* from S288C to D273-10b. Silent mutations are not indicated.

One enhanced respirer of ZV27a resulted from a novel mtDNA rearrangement

One of the enhanced respirer strains, ZV65, appeared to contain a mitochondrial mutation (Figure 5.12). In addition to causing increased respiratory growth, this mutation reduced growth in the absence of arginine relative to the parental strain ZV27a (Figure 5.13). *MSS51* is able to support growth on CSM-ARG and YPAEG in strains bearing either *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA or mtDNA from ZV65 (Figure 5.13).

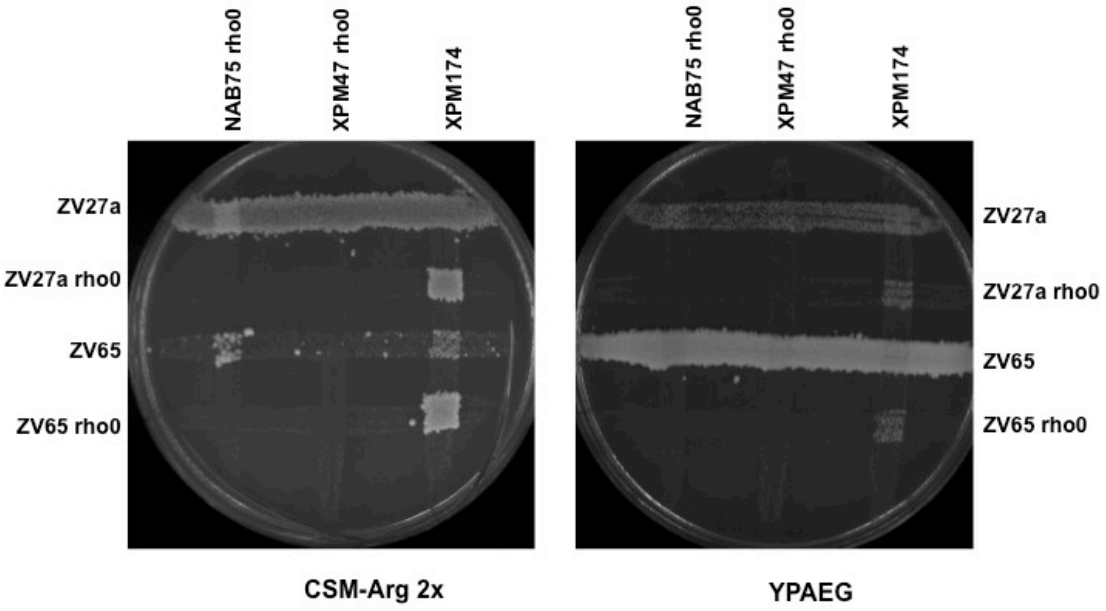

Figure 5.12 - ZV65 contains a mitochondrial suppressor that enhances the ability of *mss51-102* to support respiration. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Mated cells were printed twice to medium lacking arginine (CSM-Arg 2x) and complete ethanol/glycerol medium (YPAEG). Plates were incubated for 4 days at 30ºC. This cross has been repeated once. See Table 5.5 for complete genotypes.

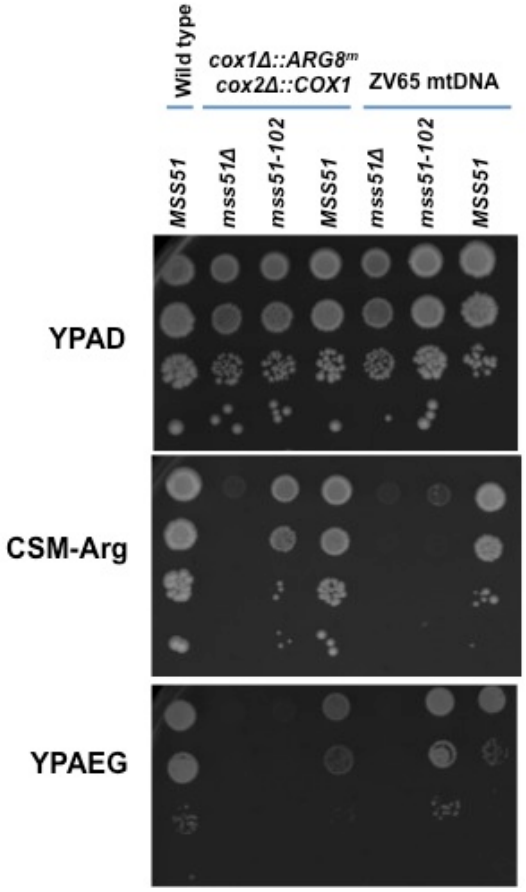

Figure 5.13 - ZV65 is largely unable to grow on medium lacking arginine, but respires strongly. Ten-fold serial dilution of cells were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. This dilution series has been repeated once. Strains bearing wild type mtDNA were: *MSS51,* DAU1. Strains bearing *cox1Δ::ARG8*^{m} *cox2Δ::COX1* mtDNA were: *mss51Δ*, XPM174; *mss51-102*, ZV27; *mss51-103,* ZV22*; MSS51,* XPM171. Strains bearing ZV65 mtDNA were: *mss51Δ*, ZV66; *mss51-102*, ZV65; *MSS51,* ZV67. See Table 5.5 for complete genotypes.

The enhanced respiration of ZV65 was found to be caused by a rearrangement of the *cox1Δ::ARG8^m cox2Δ::COX1* mitochondrial genome. I demonstrated via PCR analysis that the ZV65 mitochondrial rearrangement contains the *COX1* 5' UTR upstream of both *COX1* **and** *ARG8^m* (Figure 5.14), while ZV65 retains the parental configuration of the *COX1* 3' UTR downstream of *ARG8^m* and the *COX2 3'* UTR downstream of *COX1* (Figure 5.15). The junction between the *COX1 5' UTR* and *ARG8^m* was sequenced and I found that the junction between *COX1* 5' UTR and *ARG8^m* showed 4 A's upstream of the AUG *COX1* start codon (Figure 5.16). The junction between *COX1* 5' UTR and *COX1* coding sequence was sequenced, and ZV65 mtDNA showed only 3 A's upstream of the AUG *COX1* start codon (Figure 5.17), which indicated that a rearrangement did indeed occur. The D273-10b background strain DAU1 and the Saccharomyces Genome Database's S288C strain show 4 A's upstream of the AUG *COX1* start (Figure 5.17).

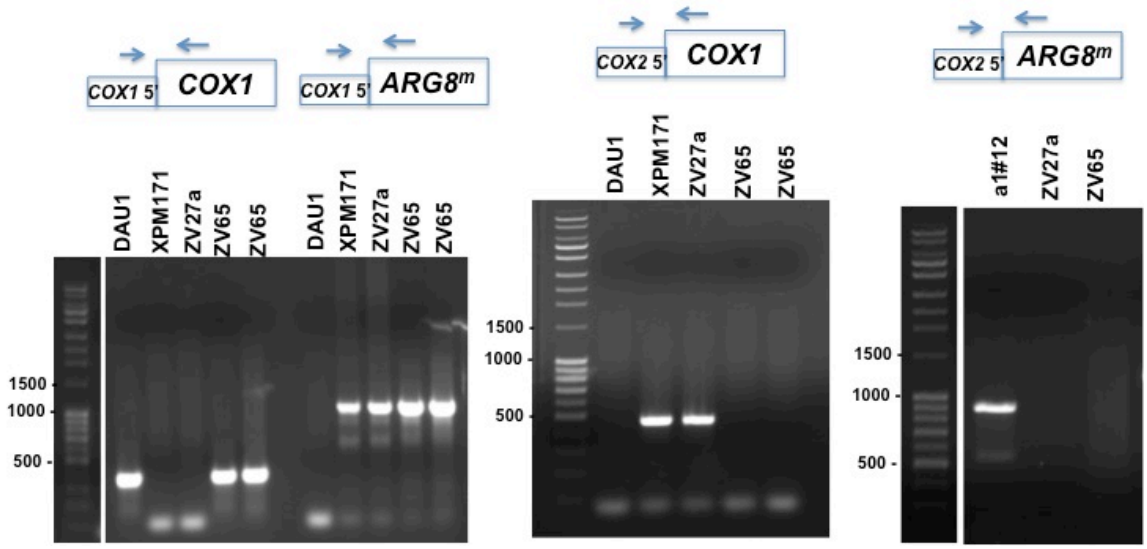

Figure 5.14 - ZV65 mtDNA contains *COX1 5' UTR* upstream of both *ARG8^m* and *COX1*. PCRs were set up to amplify the indicated mtDNA location. Products were separated on a 1% agarose gel. These PCRs have been repeated twice. Primers used are listed in the Methods and Materials. See Table 5.5 for complete genotypes.

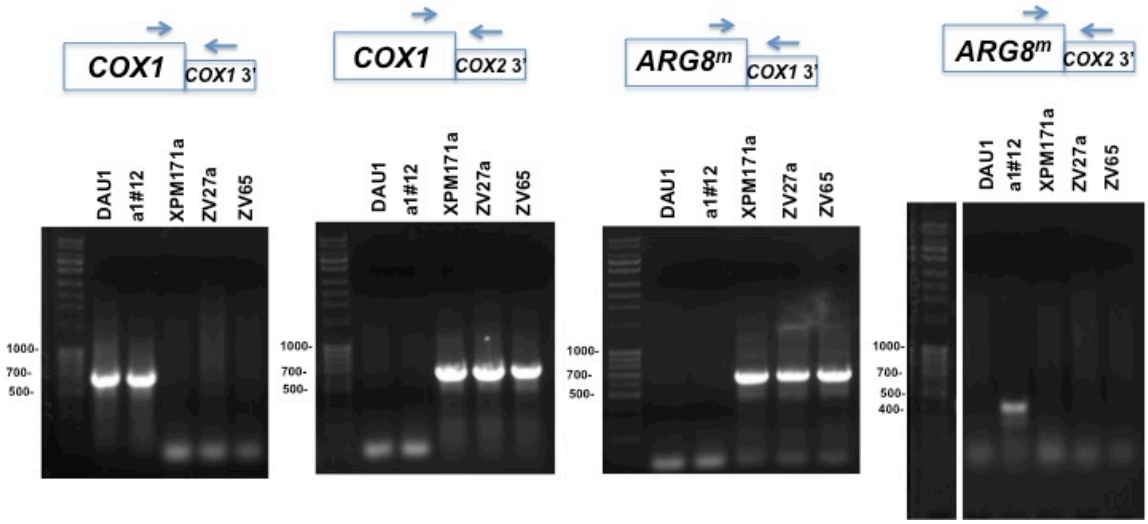

Figure 5.15 - ZV65 mtDNA retains the *COX2* 3' UTR downstream of *COX1,* and the *COX1* 5' UTR downstream of *ARG8*^{m}. PCRs were set up to amplify the indicated mtDNA location. Products were separated on a 1% agarose gel. These PCRs have been repeated twice. Primers used are listed in the Methods and Materials. See Table 5.5 for complete genotypes.

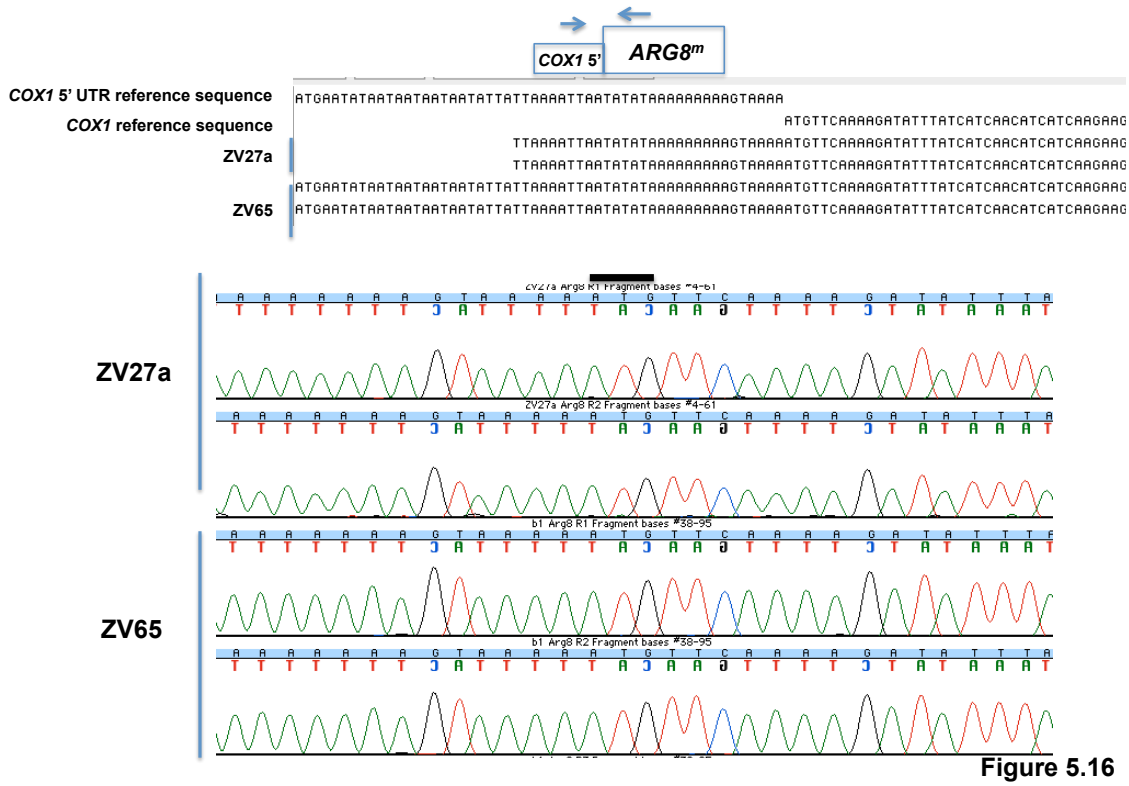

Figure 5.16

Figure 5.16 - ZV65 mtDNA retains its parental ZV27a junction between *COX1* 5' UTR and *COX1*. The *COX1 5' UTR::ARG8*^{m} PCR products from strains ZV27a and ZV65 shown in Figure 14 were sequenced by Sanger sequencing. The *COX1* start codon is indicated by the solid black bar. These sequences have been repeated twice. See Table 5.5 for complete genotypes.

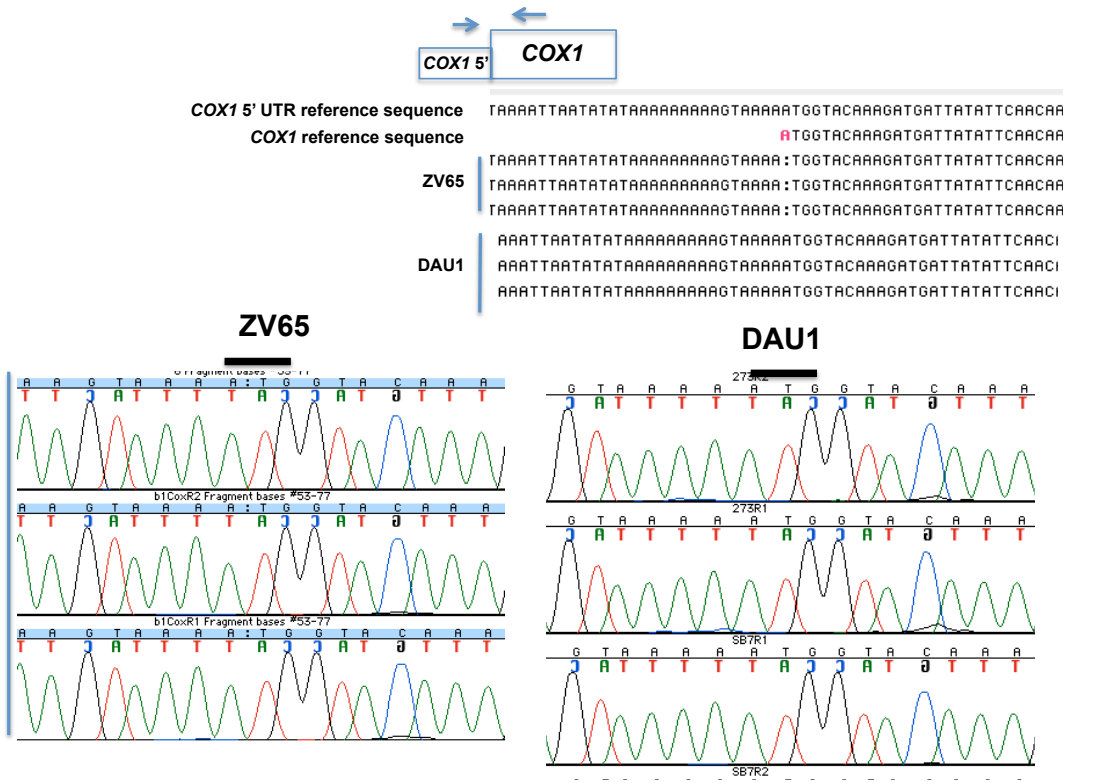

Figure 5.17

Figure 5.17 - ZV65 mtDNA lacks an 'A' preceding of the *COX1* AUG start at the *COX1* 5' UTR – *COX1* junction. The *COX1 5' UTR::COX1* PCR products from strains ZV65 and DAU1 shown in Figure 14 were sequenced by Sanger sequencing. The *COX1* start codon is indicated by the solid black bar. These sequences have been repeated twice. See Table 5.5 for complete genotypes.

Heteroplasmic mtDNA does not seem to be a likely explanation for the mitochondrial rearrangement, as it has been previously found that strains with heteroplasmic mtDNA are relatively unstable (Muller, Reif et al. 1984). The rearranged mtDNA is quite stable – after being restreaked twice to YPAD, only approximately 2% of colonies were unable to respire (Figure 5.18). When mated to NAB75 rho0 (*MSS51 arg8Δ* rho0 strain), the resulting *mss51-102/MSS51* diploids still uniformly failed to become Arg+, although several Arg+ revertants were observed (Figure 5.18). Strain ZV67 (*MSS51,* ZV65 mtDNA) had equally stable mtDNA when compared with ZV65 (Figure 5.19).

The inability of the NAB75 rho0/ZV65 diploid to grow on medium lacking arginine was surprising. To ascertain if this inability was due to a failure of ZV65 to completely mate with NAB75 rho0, I selected and restreaked diploids of NAB75 rho0 x ZV65 on SD+URA+ARG, and found that the ZV65/NAB75rho0 purified diploid strain behaved in the same fashion shown in Figure 5.18 (data not shown). This suggests that in a heterozygous *mss51-102/MSS51* diploid bearing ZV65 mtDNA, *mss51-102* is dominant to *MSS51* in the action of translation activation at the *COX1* 5' UTR leader upstream of $ARG8^{m}$, which is remarkable and unexpected.

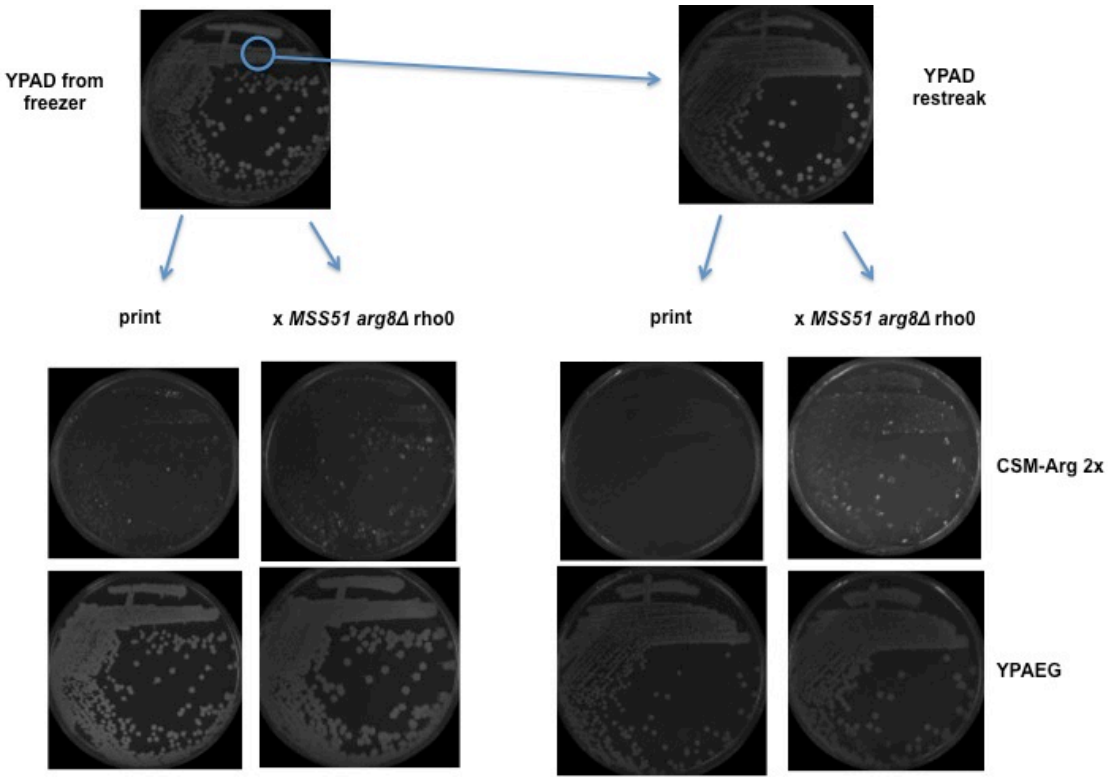

Figure 5.18 – ZV65 contains relatively stable mtDNA. ZV65 (*mss51-102*, rearranged mtDNA) was streaked to complete medium (YPAD) from a freezer stock, printed to medium lacking arginine twice (CSM-Arg 2x), and complete ethanol/glycerol medium (YPAEG). ZV65 was mated to a lawn of NAB75rho0 (*MSS51 arg8Δ* rho0) on YPAD, allowed to mate, then printed to CSM-Arg twice and YPAEG. A cluster of cells from the original YPAD plate was restreaked to YPAD, and the process repeated as indicated. This experiment has been repeated once. All plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

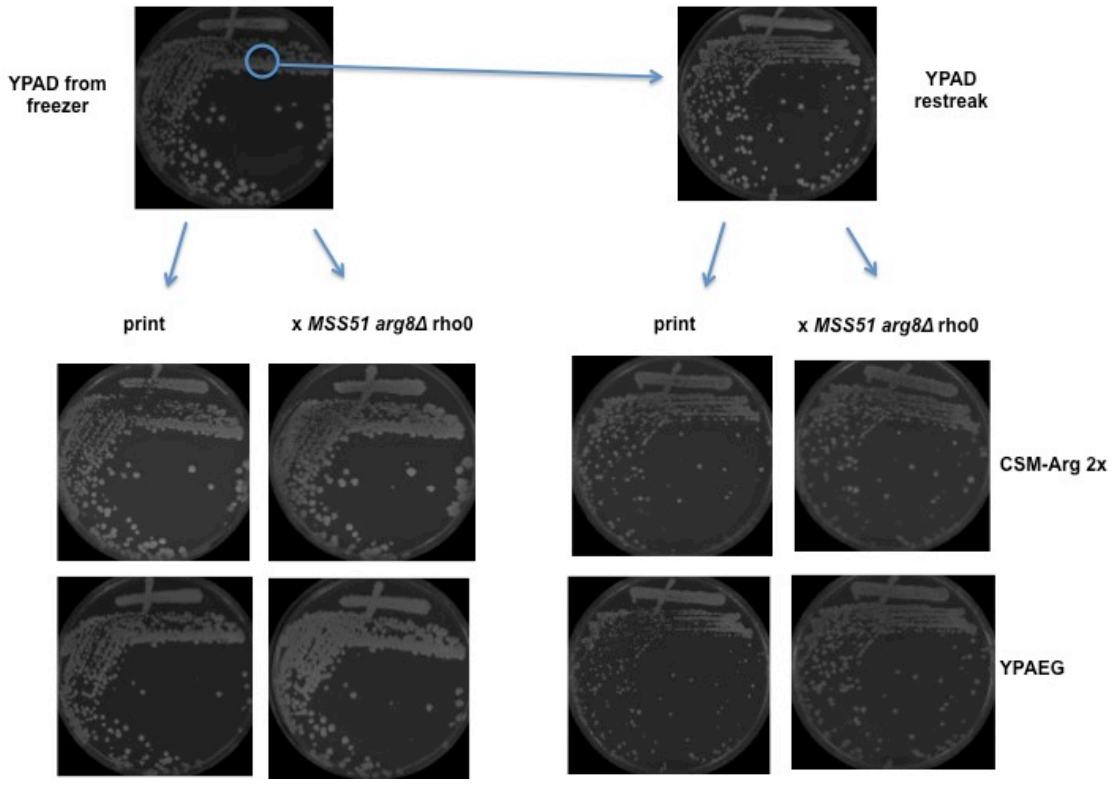

Figure 5.19 – ZV67 contains relatively stable mtDNA. ZV67 (*MSS51,* rearranged mtDNA) was streaked to complete medium (YPAD) from a freezer stock, printed to medium lacking arginine twice (CSM-Arg 2x), and complete ethanol/glycerol medium (YPAEG). ZV67 was mated to a lawn of NAB75rho0 (*MSS51 arg8Δ* rho0) on YPAD, allowed to mate, then printed to CSM-Arg twice and YPAEG. A cluster of cells from the original YPAD plate was restreaked to YPAD, and the process repeated as indicated. This experiment has been repeated once. All plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

The UTRs of *COX1* and *COX2* in a strain bearing ZV65 mtDNA have undergone extensive rearrangement

Purified mtDNA was prepared from strains ZV27a and ZV65, and each mtDNA preparation was digested with MspI (Invitrogren) (Figure 5.20). MspI restriction sites do not occur within the *COX1*, *COX2* or *ARG8*^{m} coding sequences. Southern blots were performed on MspI cleaved purified mtDNA isolated from ZV65 using radiolabeled probes against *COX1*, *COX2* and *ARG8*^{m} (Figure 5.21). The Southern blot of Cox1 is consistent with the absence of heteroplasmy for multiple forms of Cox1. The predicted and experimentally determined sizes of the bands are shown in Table 5.3. It was not possible to predict the size of the *COX1* or *COX2* fragments in ZV65 mtDNA, as the PCR and sequencing data suggested that the rearrangement had occurred in this region. In order to more fully determine the rearrangement of mtDNA derived from ZV65, I wanted to isolate and clone mitochondrial DNA fragments containing *COX1* and *COX2*, and sequence their flanking regions.

Table 5.3 – Predicted and experimentally determined size of bands from Southern blots.

mtDNA, MspI digest	Probe	Predicted size (bp)	Experimentally determined size (bp)
ZV65	Cox1	-	3000
ZV27a	Cox1	4218	4300
ZV65	Cox2	-	4300
ZV65	Cox2	-	3000
ZV65	Cox2	-	2600
ZV65	Cox2	-	2000
ZV27a	Cox2	4218	4600
ZV65	$Arg8^m$	3004	3000
ZV27a	$Arg8^m$	3004	3000

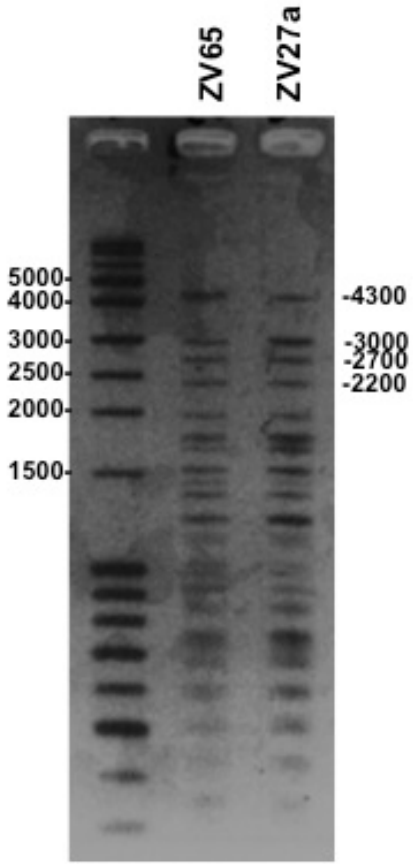

Figure 5.20 - EtBr stained agarose gel of *MspI* digested purified mtDNA from ZV65 and ZV27a shows similar patterns. Purified mtDNA was prepared as described in the Methods and Materials. 3 ug of purified mtDNA was digested with 10 U *MspI* for 2 hours at 37ºC. The reaction was electrophoresed on a 1.5 % agarose gel containing 60 ug EtBr for 4 hours at 70 V and visualized an AlphaImager Mutlimage III using FluorchemQ software. This experiment has not been repeated. See Table 5.5 for complete genotypes.

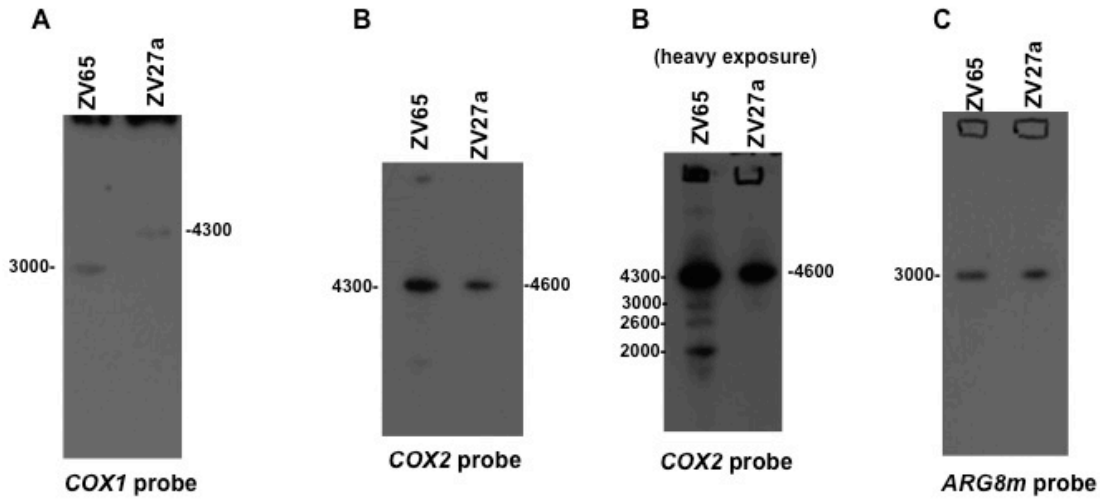

Figure 5.21 – Southern blot probes of ZV65 and ZV27a purified mtDNA show differences for *COX1* and *COX2.* Probes for (A) *COX1,* (B) *COX2* and (C) *ARG8*^{m} were designed using the primers listed in the Methods and Materials. Probes were hybridized to the membrane and the membranes were visualized by autoradiography. See Table 5.5 for complete genotypes.

For the sequencing and examination of *COX1, COX2* and their flanking regions isolated from mtDNA from ZV65, it is important to note the parent strain ZV27a bears *cox1Δ::ARG8^m cox2Δ::COX1* mtDNA, in which *COX1* is flanked by 73 and 119 bp of the *COX2* 5' and 3' UTRs and is placed 295 bp upstream of *COX2* and in the opposite orientation, with *COX2* at its native locus (Figure 5.22A). The *COX1* probe hybridized to different sized fragments of MspI digested purified mtDNA from ZV65 and ZV27a, which is an encouraging result (Figure 5.21a). I successfully cloned the band containing *COX1* from ZV65 mtDNA into the vector pBluescript II KS+, creating plasmid pZV24. The regions flanking *COX1* were sequenced from pZV24 and it was found that prior to the AUG start of *COX1* in ZV65 mtDNA, 1 kb of *COX1* 5' UTR is present. There is an MspI site 1005 bp upstream of the *COX1* start in the wild type reference sequence, so I was unable to glean any additional information concerning the mtDNA preceding the *COX1* AUG start. Following the *COX1* TAA stop I found 119 bp of the *COX2* 3' UTR, followed by 296 bp of the *COX2* upstream region (Figure 5.22B). The region following the *COX1* TAA stop is the same in the parent ZV27a and in the rearranged ZV65 mtDNA. The *COX1 MspI* fragment from ZV65 mtDNA differs from the *COX1 MspI* fragment from ZV27a mtDNA in that the *COX2* 5' UTR has been replaced in ZV65 with at least 1 kb of the *COX1* 5' UTR immediately preceding the *COX1* AUG start, and the deletion of a single 'A' immediately upstream of the AUG start.

The *COX2* probe hybridized to a fragment of approximately 4.5 kb of MspI digested purified mtDNA from ZV27a, which matches roughly with the predicted fragment of 4.2 kb. A heavy exposure of the *COX2* probe shows that the *COX2* probe

hybridized to a total of 4 bands of MspI digested purified mtDNA from ZV65 (Figure 5.21C). It is not readily apparent why multiple bands in ZV65 mtDNA should be detected with a *COX2* probe. I cloned the predominant band containing *COX2* from the digest of purified mtDNA from ZV65 (the largest band ZV65 lane in from Figure 5.20) into the vector pBluescript II KS+ creating plasmid pZV25 and sequenced the flanking regions.

pZV25 contains *COX2* with no additional mutations from the D273 *COX2* sequence. The TAA stop of *COX2* is followed by 1134 bp of the *COX2* 3' flanking sequence, as expected. Preceding the *COX2* AUG start is a complex and as-yet not fully sequenced region. As shown in Figure 5.22C, immediately upstream of the *COX2* AUG start is 75 bp of the *COX2* 5' UTR. Upstream of the *COX2* 5' UTR is a region for which I have been unable to acquire reliable sequence data, despite rigorous efforts to do so. 800 bp of the *COX2* 3' UTR is **also** present upstream of the *COX2* AUG start in pZV25, in the opposite orientation of the rest of the *COX2* message and flanking region (Figure 2.2C). As best I can tell, the additional copy of the *COX2* 3' UTR did not result from the ligation of an incomplete digest, as the insert fragment of pZV25 does not contain an *MspI* site (data not shown).

The Southern probed with *ARG8*^{m} matched expectations - based on sequencing of the PCR product, no change in either the *COX1* 5' and *3'* UTR flanking *ARG8*^{m} was detected between ZV27a and ZV65 (Figure 5.21c). I predicted that a 3 kb fragment would be found in each case, and I observed the expected sized fragment. I was unable to detect any mitochondrial rearrangements that altered the

configuration *cox1Δ::ARG8^{m}* at the *COX1* locus. I did not clone a mtDNA fragment containing *ARG8^{m}* into a plasmid vector.

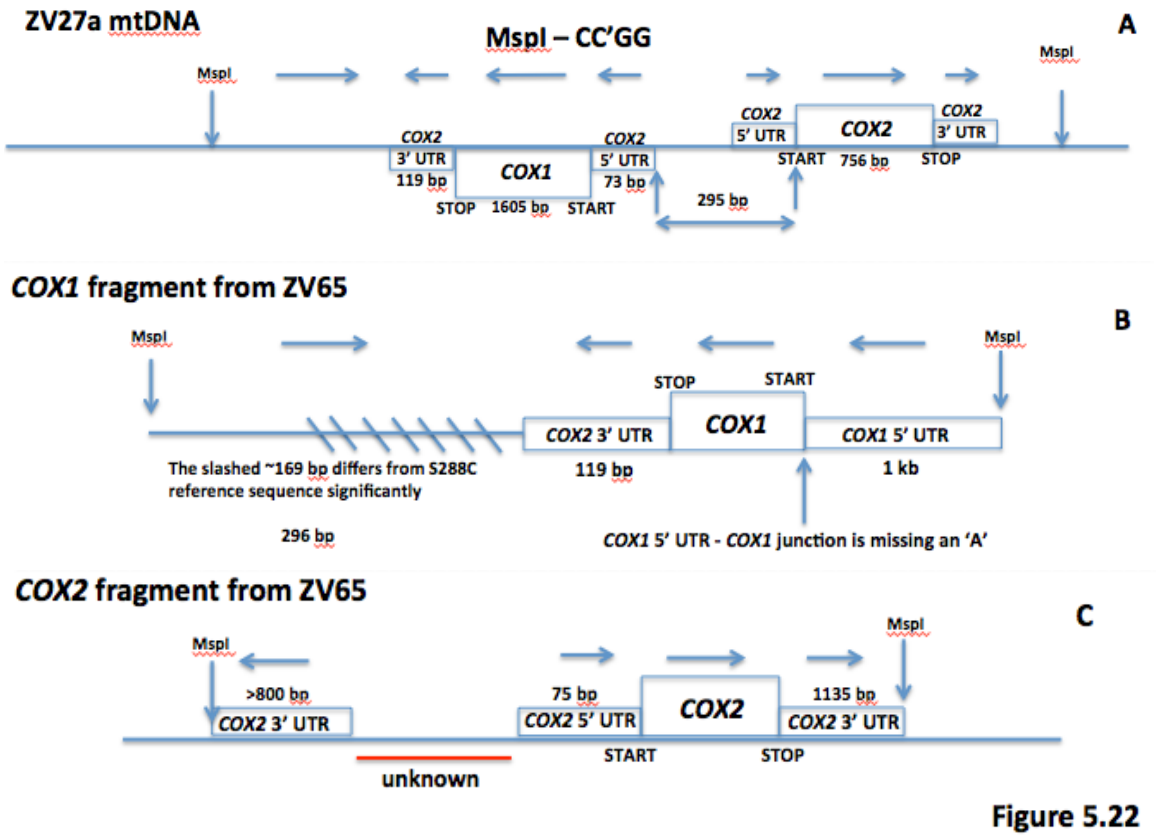

Figure 5.22 – Graphical representations of mtDNA fragments from ZV27a and ZV65.

ZV65 is unable to support arginine biosynthesis due to a quantitative effect of mss51-102p

As previously discussed, the arginine phenotype of ZV65 is surprisingly Arg-, although Arg+ revertants can be obtained at high frequency. To test if the *ARG8^{m}* gene was active in the rearranged mtDNA, *MSS51* was crossed into a strain with ZV65 mtDNA, and it was found that *MSS51* in ZV65 mtDNA was Arg+ and Pet+, so the *ARG8m* gene is functional (Figure 5.23). Thinking that perhaps the Arg- phenotype was influenced by an additional mutation in *mss51-102*, I sequenced *mss51-102* from ZV65 and found that it was unchanged from the parent. To test if ZV65 had acquired a nuclear mutation that made it Arg-, ZV65 was made rho0 via EtBr treatment and crossed to XPM174 (*mss51Δ::LEU2, cox1Δ::ARG8^{m} cox2Δ::COX1)*. Tetrads were dissected and arginine and respiratory growth went 2:2, which meant that the *mss51-102* allele in ZV65 is capable of activating translation at the *COX1* 5' UTR, and that there are no nuclear Arg- mutations in the ZV65 (Figure 5.24).

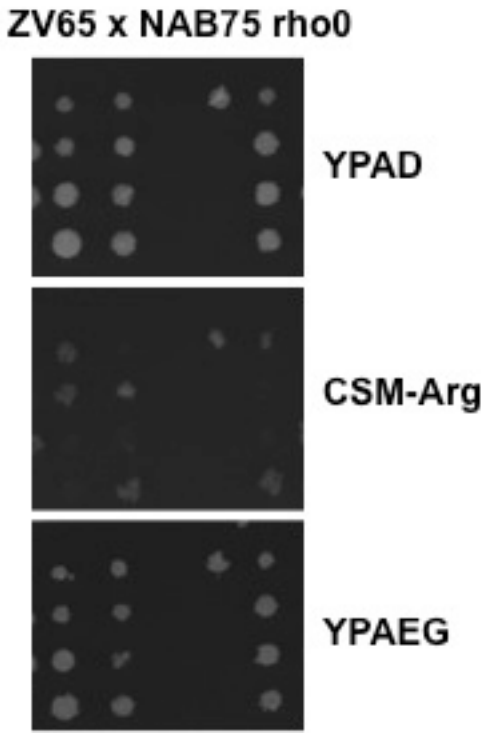

Figure 5.23 - ZV65 contains an active *ARG8^m* gene. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Diploids were selected, sporulated, and tetrads were dissected on YPAD. Spores were printed twice to medium lacking arginine (CSM-Arg 2x) and complete ethanol/glycerol medium (YPAEG). Plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

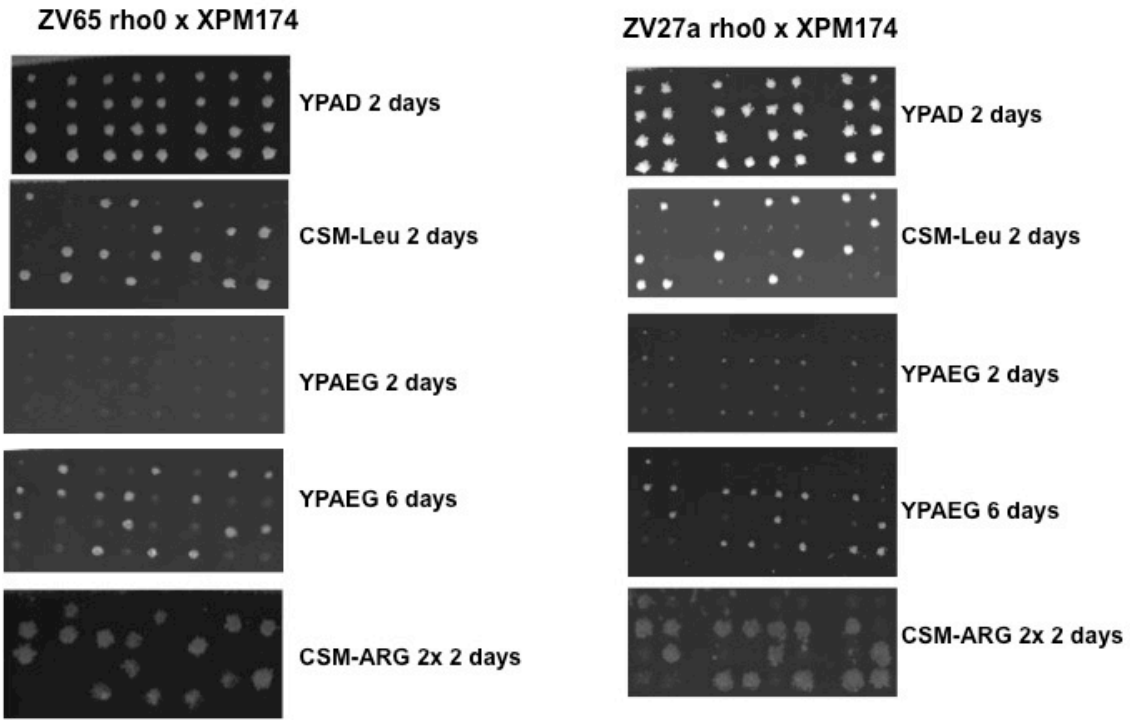

Figure 5.24 - *mss51-102* in ZV65 can activate *ARG8*^{m} translation at *COX1* 5' UTR, and ZV65 contains no nuclear arginine mutations. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Diploids were selected, sporulated, and tetrads were dissected on YPAD. Spores were printed to medium lacking leucine (CSM-Leu), complete ethanol/glycerol medium (YPAEG), and twice to medium lacking arginine (CSM-Arg 2x). Plates were incubated for 2 or 6 days at 30ºC as indicated. See Table 5.5 for complete genotypes.

As the *ARG8^{m}* gene is active, and *mss51-102* is capable of activating translation at the *COX1* 5' UTR, one possible explanation for why *mss51-102* was Arg- in ZV65 mtDNA is a quantitative effect of mss51-102p. *mss51-102* and *MSS51* were overexpressed on 2u vectors in strain ZV65. I observed that Arg growth increased, but still not to WT levels (Figure 5.25). Based on this, the most likely explanation for why *mss51-102* is Arg- in a strain bearing ZV65 mtDNA is a dosage effect, and when the 5' UTR is present in two locations, *mss51-102* is preferentially involved in the assembly of cytochrome *c* oxidase, not in translational activation at the *COX1* 5' UTR.

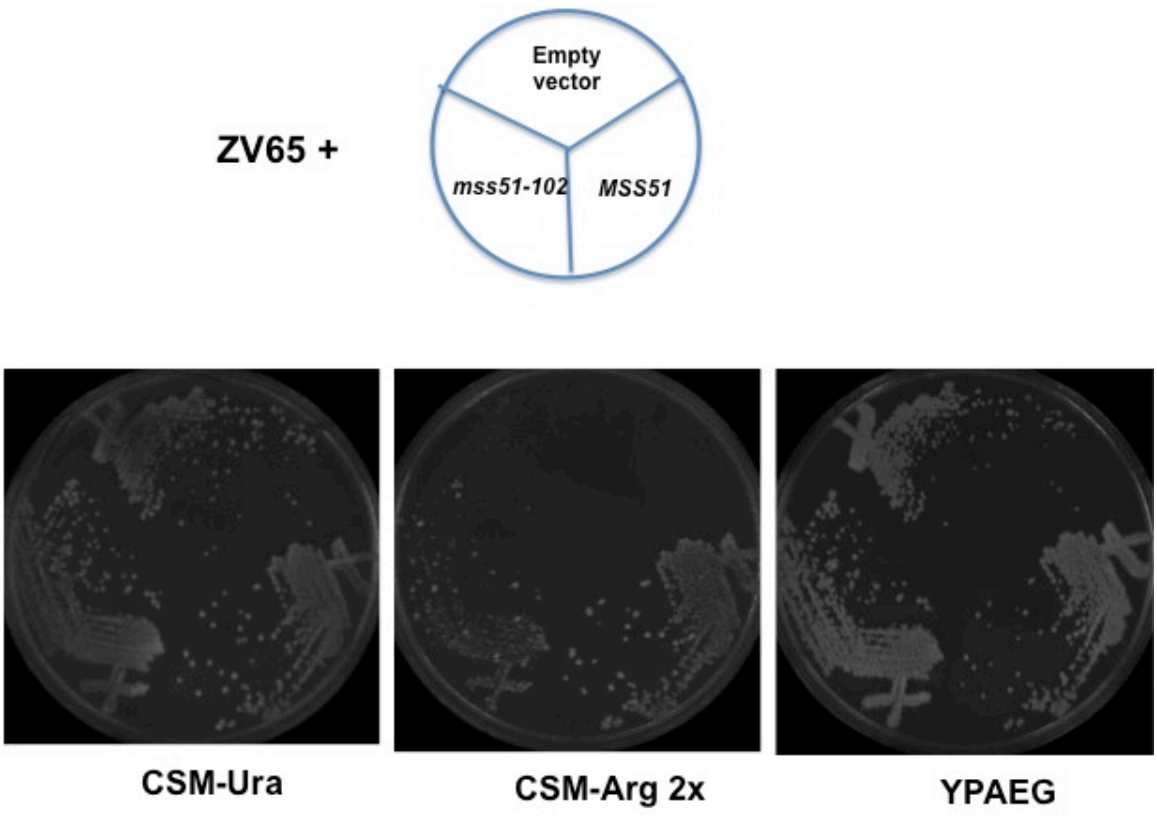

Figure 5.25 - The inability of ZV65 to grow on medium lacking arginine is likely due to a quantitative effect of *mss51-102.* Strain ZV65 was transformed with either: empty vector, YEp352; *mss51-102,* pZV23; *MSS51,* pZV18. Transformants were streaked on medium lacking uracil (CSM-Ura), then printed twice to medium lacking arginine (CSM-Arg 2x) and to complete ethanol/glycerol medium (YPAEG). This experiment has been repeated once. Plates were incubated for 2 days at 30ºC. See Table 5.5 for complete genotypes.

Arg+ revertants of ZV65 can be found at high frequency

I selected 4 independent Arg+ revertants of ZV65, termed *argmut-1, argmut-2, argmut-3, argmut-4* (Figure 5.26A). In each Arg+ revertant, respiratory growth was decreased slightly relative to its parent. Arg+ revertants *argmut-1, argmut-2, argmut-3, argmut-4* were crossed to ZV18b rho0 (*mss51-102 ARGMUT,* rho0) and the resulting diploids were Arg-, so the Arg+ mutation is nuclear recessive in all 4 arginine revertants (Figure 5.26A). Complementation analysis was performed on the four Arg+ revertants, and it was found that a diploid of each combination (*argmut-1/ argmut-2, argmut-1/ argmut-3, argmut-1/ argmut-4, argmut-2/ argmut-3, argmut-2/ argmut-4, argmut-3/argmut-4*) was Arg+ in each case, indicating that while these four revertants were derived independently, they fail to complement and therefore each have a mutation in the same gene (Figure 5.26B). When tetrads of the diploids were dissected, I found that in each arginine revertant, a single nuclear recessive mutation was responsible for the Arg+ phenotype (Figure 5.27). I tested to see if the recessive mutations were caused by a reversion in the nuclear *arg8::hisG* gene by making the Arg+ strains rho0 via 2x EtBr treatment and examining their arginine phenotype. All four arginine revertants were unable to grow on media lacking arginine lacking mtDNA, so *arg8::hisG* did not revert (data not shown). The Arg+ revertants do not show any temperature sensitivity for respiratory growth relative to ZV67 (*MSS51,* ZV65 mtDNA) at 14ºC, 16ºC, 20ºC, 25ºC, 30ºC, 32ºC, or 37ºC (data not shown). The identity of the recessive mutation remains unknown, although I sequenced *mss51-102* in each Arg+ revertant and found that no additional mutations occurred.

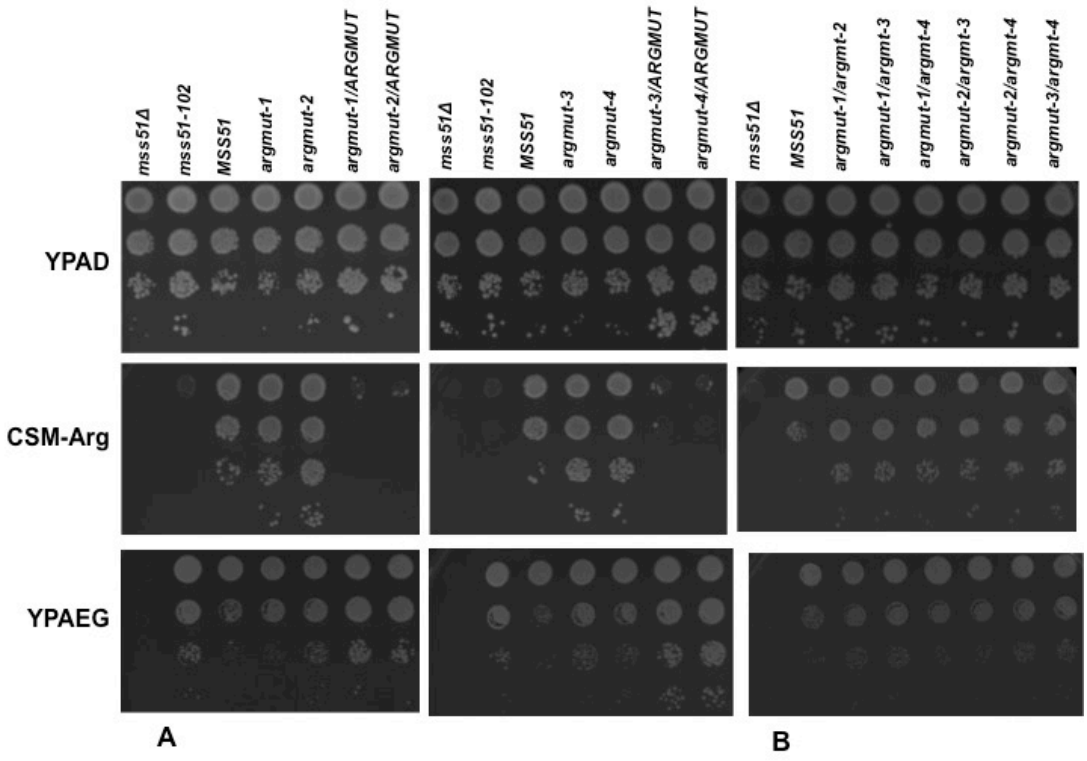
mss51Δ
mss51-102
MSS51
argmut-1
argmut-2
argmut-1/ARGMUT
argmut-2/ARGMUT
mss51Δ
mss51-102
MSS51
argmut-3
argmut-4
argmut-3/ARGMUT
argmut-4/ARGMUT
mss51Δ
MSS51
argmut-1/argmt-2
argmut-1/argmt-3
argmut-1/argmt-4
argmut-2/argmt-3
argmut-2/argmt-4
argmut-3/argmt-4
YPAD
CSM-Arg
YPAEG
A
B

Figure 5.26 – Arg+ revertants of ZV65 can be identified. (A) 4 independent Arg+ revertants of ZV65 were identified in strains bearing rearranged ZV65 mtDNA, and each revertant mutation is nuclear recessive. Ten-fold serial dilution of cells were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. All strains contain the rearranged ZV65 mtDNA. This dilution series has not been repeated. Strains were: *mss51Δ*, ZV66; *mss51-102,* ZV65; *MSS51,* ZV67; *argmut-1*, ZV89; *argmut-2*, ZV90; *argmut-1*/*ARGMUT*, ZV93; *argmut-2*/*ARGMUT*, ZV94; *argmut-3*, ZV91; *argmut-4*, ZV92; *argmut-3*/*ARGMUT*, ZV95; *argmut-4/ARGMUT*, ZV96. See Table 5.5 for complete genotypes. (B) Complementation analysis of 4 Arg+ revertants of ZV65 show the arginine reversion is caused by mutations in the same gene. Ten-fold serial dilution of cells were spotted on complete medium (YPAD), medium lacking arginine (CSM-Arg) and complete ethanol/glycerol medium (YPAEG) and incubated for 3 days at 30ºC. All strains contain the rearranged ZV65 mtDNA. This dilution series has not been repeated. Strains were: *mss51Δ*, ZV66; *MSS51,* ZV67; *argmut-1*/*argmut-2* ZV97; *argmut-1*/*argmut-3*, ZV98; *argmut-1/argmut-4*, ZV99; *argmut-2/argmut-3*, ZV100; *argmut-2*/*argmut-4*, ZV101; *argmut-3/argmut-4*, ZV102. See Table 5.5 for complete genotypes.

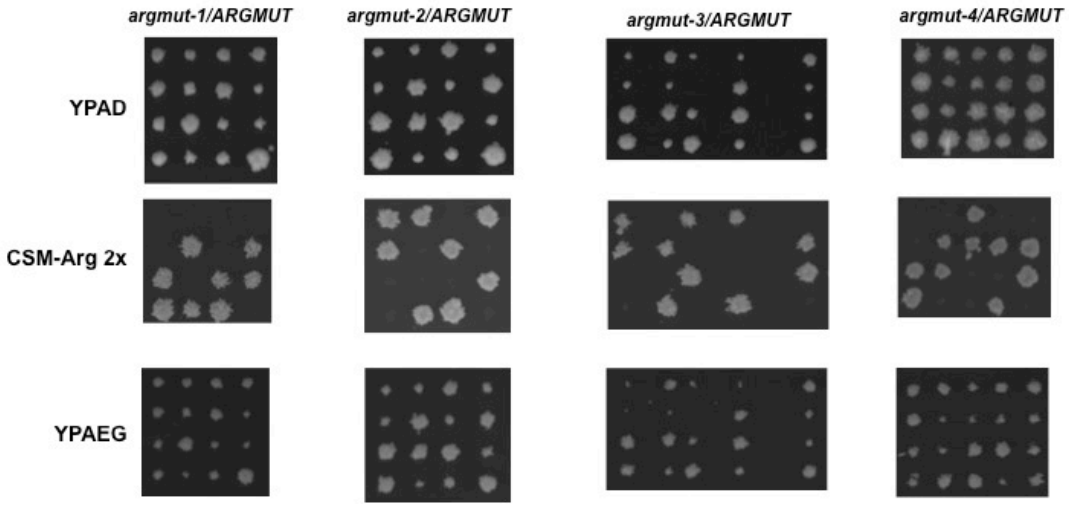

Figure 5.27 - 4 independent Arg+ revertants of ZV65 each contain a single suppressor mutation. Haploid cells of the indicated strains were patched in vertical stripes. The stripes were cross-printed on complete medium (YPAD) and allowed to mate. Diploids were selected, sporulated, and tetrads were dissected on YPAD. Spores were printed twice to medium lacking arginine (CSM-Arg 2x) and complete ethanol/glycerol medium (YPAEG). Plates were incubated for 3 days at 30ºC. All strains contain the rearranged ZV65 mtDNA. Strains were: *argmut-1/ARGMUT*, ZV93; *argmut-2/ARGMUT*, ZV94; *argmut-3/ARGMUT*, ZV95; *argmut-4/ARGMUT*, ZV96. See Table 5.5 for complete genotypes.

DISCUSSION

The majority of experiments on this class of mutants were only performed using *mss51-102*. *mss51-103* shows a similar phenotype to *mss51-102* and I predict that *mss5-103* would behave in a similar fashion to *mss51-102*, but this has not been tested experimentally.

I propose that *mss51-102* and *-103* are able to strongly support respiration when the *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence - in strains bearing either wild type or *COX1(1-512)::ARG8^{m}* mtDNA. However, when the *COX1* 5' UTR is in *trans* to the *COX1* coding sequence, as in *cox1Δ::ARG8^{m} cox2Δ::COX1* mtDNA, *-102* and *-103* are impaired at moving from the *COX1* mRNA 5' UTR to the newly synthesized Cox1 protein and respiratory growth is severely abrogated. Taken together, these data suggest that binding of an Mss51-102 or Mss51-103 molecule to the *COX1* 5'-UTR *cis* to the *COX1* coding sequence greatly facilitates its interaction with newly synthesized Cox1 emerging from ribosomes translating the same mRNA molecule to which it was bound. By extension, I propose that wild-type Mss51 molecules also preferentially act in *cis* with the wild-type *COX1* mRNA, despite their previously established ability to act in *trans* to promote cytochrome *c* oxidase assembly in strains containing the chimeric mRNA (Figure 5.28). This is a previously undescribed and biologically relevant function of Mss51p. Our hypothesis of the *cis*/*trans* nature of *mss51-102* and *-103* is supported by the spontaneous rearrangement of the mtDNA found in ZV65. ZV65 mtDNA copied the *COX1* 5' UTR leader in *cis* to the *COX1* coding sequence, and *mss51-102* respires at

near WT levels as a result. This is reassuring biological confirmation of our hypothesis.

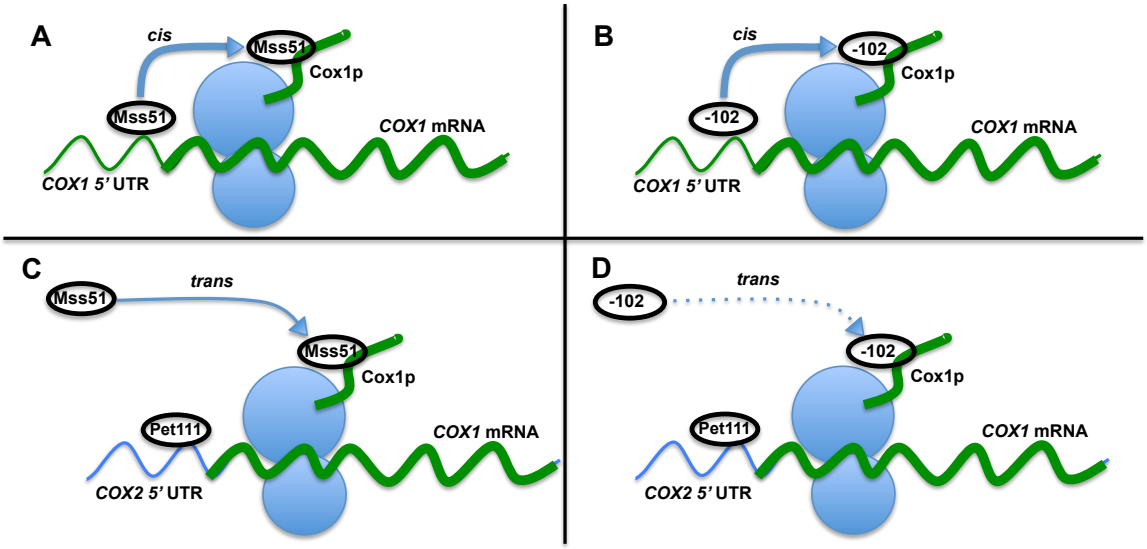

Figure 5.28 - A *cis/trans* model for the function of Mss51 and Mss51-102. (A, B) When the *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence (as in strains bearing wild type mtDNA), both Mss51 and Mss51-102 are capable of activating translation at the *COX1* 5' UTR and interacting with nascent Cox1, resulting in strong levels of growth on complete ethanol/glycerol (YPAEG) medium. (C) When the *COX1* 5' UTR is present in *trans* to the *COX1* coding sequence (as in strains bearing *cox1Δ::ARG8m cox2::COX1* mtDNA), Mss51 can move from the *COX1* 5' UTR to nascent Cox1, although growth on complete ethanol/glycerol (YPAEG) medium is reduced relative to growth when the *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence. (D) When the *COX1* 5' UTR is present in *trans* to the *COX1* coding sequence (as in strains bearing *cox1Δ::ARG8m cox2::COX1* mtDNA), Mss51-102 is highly reduced in its ability to move from *COX1* 5' UTR to nascent Cox1, resulting in very low levels of growth on complete ethanol/glycerol (YPAEG) medium.

This proposed *cis* function of *MSS51* could also provide an explanation for why I was unable to identify any *mss51* mutants that were Arg- and Pet+ in the

initial mutant screen discussed in Chapter 2. As the screen was performed in strain XPM174, an Arg- but Pet+ phenotype would indicate that the *mss51* allele would be capable of targeting the *COX1* coding sequence without first activating translation at the *COX1* 5' leader. If the interaction with the *COX1* 5' leader was somehow necessary for, or significantly facilitated the ability of Mss51 to target the *COX1* coding sequence, it would be exceedingly difficult to find an *mss51* allele which is incapable of interacting the leader but could interact with newly synthesized Cox1p.

It should be noted that when sequencing the plasmid containing the *COX1* fragment from ZV65 mtDNA, I found that 169 bp of the region upstream of the native *COX2* locus (shown in slashed lines in Figure 5.22B) was highly divergent between the D273-10b strain background sequence and the SGD S288C reference sequence. I amplified and sequenced this region from a PCR product of DAU1 genomic DNA and found that it matched the fragment on the plasmid. The reason for the sequence divergence between D273-10b and S288C background strains is unclear. For reference, the sequence of D273-10b *COX2* with 548 bp 5' UTR and 1124 bp 3' UTR flanking regions is included in Table 5.4.

It is not clear why I have been unable to fully sequence the region upstream of the *COX2* AUG start in pZV25. I have tried multiple reverse primers internally within *COX2* and the sequence reads all fail within ~75 bp upstream of the *COX2* AUG start. It is also unclear as to what mitochondrial rearrangement could have occurred to result in the mitochondrial genome I identified in ZV65. There appears to be extensive recombination around both *COX1* and *COX2*, involving their respective UTR's. It would be a more complete story if the recombination event could be drawn out, and furthermore if I could show where *COX1* is in the genome, relative to *COX2*. As it currently stands I do not know if *COX1* has moved away from its previous location 295 bp upstream of the *COX2* loci. Regardless, ZV65 mtDNA is a powerful validation of the model that mss51-102p respires more strongly when the *COX1* 5' UTR is present in *cis* to the *COX1* coding sequence.

Table 5.4 - *COX2* sequence with UTRs from D273-10b strain background. The *COX2* sequence has 548 bp 5' UTR and 1124 bp 3' UTR sequence. The ATG and TAA start and stop are bold and underlined.

GGCGGGGACCCCGAAGGAGTATAAATAAAAATTAATAATATATTATATATATATTATATTAATAATAATAATAATAATAAATAAT

AACTCCTTGCTTCATACCTTTATAATAATAATAATTATATTTATAATTTATATATTTATATATATAAAAATTATATATATTATATATAA

TCTAAATATTATATATTTTAATAAATATTAATATATATGATATGAATATTATTAGTTTTCGGGAAGCGGGAATCCCGTAAGGAGTGAGG

GACCCCTCCCTATACTAACGGGAGGGGGACCGAACCCCGAAGGAGTTTTATTTTTAGTATTTTATAAAATATATATTTATATGATTAAT

AATATTATATATATTATTTATAAAAATAATATATAATTTTAATTATTTTTAATAAAAAAAGGTGGGGTTTGGTAATATAATCTCTTTAT

TCTATTTATAATATATAATAATAAATTATAAATAAATTTTAATTAAAAGTAGTATTAACATATTATAAATAGACAAAAGAGTCTAAAG

GTTAAGATTTATTAAA**ATG**TTAGATTTATTAAGATTACAATTAACAACATTCATTATGAATGATGTACCAACACCTTATGCATGTTATT

TTCAGGATTCAGCAACACCAAATCAAGAAGGTATTTTAGAATTACATGATAATATTATGTTTTATTTATTAGTTATTTTAGGTTTAGTA

TCTTGAATGTTATATACAATTGTTATAACATATTCAAAAAATCCTATTGCATATAAATATATTAAACATGGACAAACTATTGAAGTTAT

TTGAACAATTTTTCCAGCTGTAATTTTATTAATTATTGCTTTCCCTTCATTTATTTTATTATATTTATGTGATGAAGTTATTTCACCAGC

TATAACTATTAAAGCTATTGGATATCAATGATATTGAAAATATGAATATTCAGATTTTATTAATGATAGTGGTGAAACTGTTGAATTTG

AATCATATGTTATTCCTGATGAATTATTAGAAGAAGGACAATTAAGATTATTAGATACTGATACTTCTATAGTTGTACCTGTAGATACA

CATATTAGATTCGTTGTAACAGCTGCTGATGTTATTCATGATTTTGCTATCCCAAGTTTAGGTATTAAAGTTGATGCTACTCCTGGTAGA

TTAAATCAAGTTTCTGCTTTAATTCAAAGAGAAGGTGTCTTCTATGGGGCATGTTCTGAGTTGTGTGGGACAGGTCATGCAAATATGCC

AATTAAGATCGAAGCAGTATCATTACCTAAATTTTTGGAATGATTAAATGAACAA**TAA**TTAATATTTACTTATTATTAATATTTTTAAT

TATTAAAAATAATAATAATAATAATAATTATAATAATATTCTTAAATATAATAAAGATATAGATTTATATTCTATTCAATCACCTTATA

TTAAAAATATAAATATTATTAAAAGAGGTTATCATACTTCTTTAAATAATAAATTAATTATTGTTCAAAAAGATAATAAAAATAATAA

TAAGAATAATTTAGAAATAGATAATTTTTATAAATGATTAGTAGGATTTACAGATGGAGATGGTAGTTTTTATATTAAATTAAATGAT

AAAAAATATTTAAGATTTTTTTATGGTTTTAGAATACATATTGATGATAAAGCATGTTTAGAAAAGATTAGAAATATATTAAATATAC

CTTCTAATTTTGAAGAACTACTTAAAACAATTATATTAGTAAATTCACAAAAGAAATGGTTATATTCTAATATTGTAACTATTTTTGAT

AAGTATCCTTGTTTAACAATTAAATATTATAGTTATTATAAATGAAAAATAGCTATAATTAATAATTTAAATGGTATATCTTATAATAA

TAAAGATTTATTAAATATTAAAAATACAATTAATAATTATGAAGTTATACCTAATTTAAAAATTCCATATGATAAAATAAATGATTAT

TGAATTTTAGGTTTTATTGAAGCTGAAGGTTCATTTGATCTATCTCCAAAACGTAATATTTGTGGTTTTAATGTTTCACAACATAAACG

TAGTATTAATACATTAAAAGCTATTAAATCTTATGTATTAAATAATTGAAAACCAATTGATAATACACCATTATTAATTAAAAATAAAT

TATTAAAAGATTGAGATTCATCTATTAAATTAACTAAACCTGATAAAAATGGAGTTATTAAATTAGAATTTAATAGAATAGATTTTTTA

TATTATGTTATTTTACCTAAATTATATTCATTAAAATGATATAGTCGTAAAGAAATTGATTTCCAATTATGAAAAACACTTATAGAAAT

CTATATAAAAGGTTTACATAATACACTTAAAGGTTCTAATTTATTAAAATTAATTAATAATAATATTAATAAAAAAAGATATTATTCTA

ATTATAATATTTCTCCTTTCGGGGTTCCG

I compared the sequence of *MSS51* provided from the S288C reference strain on Saccharomyces Genome Database to our sequence of *MSS51* from the D273-10b background and found 5 silent and 3 missense mutations between the two strain backgrounds. As *MSS51* has only 436 amino acids, a change of 3 amino acids could potentially be significant. None of these three amino acids were ever altered in the various *mss51* alleles I identified, however. These changes are represented in Figure 5.11 and Table 5.2.

The Arg- phenotype of an *mss51-102/MSS51* diploid bearing ZV65 mtDNA observed in Figure 5.18 is striking. Its not clear how Mss51-102p could stop Mss51p from activation translation at the *COX1* 5' UTR upstream of $ARG8^{m}$ in ZV65 mtDNA. Lindsay Burwell demonstrated that Mss51p does not interact with itself, so I consider it unlikely that Mss51-102p would somehow bind to or sequester Mss51p. Determining the identity of the mutation responsible for the Arg+ revertants of ZV65 might assist in explaining the unusual growth on CSM-Arg of ZV65. It is unfortunate that the mutations do not show temperature sensitivity on YPAEG medium, as that would greatly facilitate finding the gene. Its not clear to me whether Illumina/Solexa sequencing would be worth doing on these mutants; four independent revertants all with mutations in the same gene should cause the sequence analysis to be easier, but it would be an expensive project.

Determining the identity of the nuclear recessive mutation *argmut*, which restores arginine growth to strain ZV65 (*mss51-102*, *cox1Δ::ARG8m, cox1::COX1::cox2, COX2* mtDNA) could provide biologically relevant information concerning the function of

MSS51. As Mss51p is thought to be at or near rate limiting levels in the cell (Perez-Martinez, Butler et al. 2009), it is possible that ZV65 is unable to grow on medium lacking arginine because *mss51-102* is slow at departing from the intermediate assembly complexes and therefore preferentially supports respiratory growth over growth on medium lacking arginine in a strain bearing ZV65 mtDNA. If this hypothesis were correct, *argmut* could be a loss of function of a Cox1 assembly factor like Shy1 or Coa1. In the D273-10b strain background, the Fox lab has observed that either a *shy1Δ* or *coa1Δ* does not result in the loss of respiration. If the deletion of such a factor could cause mss51-102p to dissociate more readily from the intermediate assembly complex, it would free some mss51-102p to activate translation at the *COX1* 5' UTR upstream of $ARG8^{m}$, allowing for growth on medium lacking arginine. This hypothesis is supported by the fact that when arginine growth is increased in the *argmut* strains (*argmut-1*, ZV89; *argmut-2*, ZV9; ZV91; *argmut-4*, ZV92) respiratory growth is decreased, which suggests that some mss51-102p could be shifted from its role in interacting with Cox1p to its role in activating translation at *cox1Δ::ARG8m*.

In order to identify *argmut*, I would transform any of the four *argmut* strains listed above – as each strain contains a single recessive mutation and the four strains fail to complement, therefore the mutation is inn the same gene – and transform it with the plasmid library from the Prelich lab (Jones, Stalker et al. 2008) (Open Biosystems). I would select for transformants on complete ethanol/glycerol medium lacking leucine (CSM-Leu EG), which would select for the plasmid while eliminating rho- transformants. Transformants would be selected at a relatively low

density so that individual colonies could be determined (approximately 200 per plate) and printed to medium lacking arginine (CSM-Arg). A desired transformant would be Arg-. I would then determine if the presence of the plasmid was genetically linked to the Arg- phenotype by restreaking the Arg- transformant to YPAD medium and printing to CSM-Leu, screening for colonies that have spontaneously lost the plasmid. I would then print these colonies to CSM-Arg to determine if the loss of the plasmid restored the ability to grow on CSM-Arg. Once I identified plasmids that were genetically linked to the inability to grow on CSM-Arg, I would isolate the plasmids from yeast, transform them into *E. coli* and sequence the insert.

An additional project I would like to attempt would be a repeat of the selection of enhanced respirers, but for allele *mss51-103* in a strain bearing *cox1Δ::ARG8m, cox2::COX1* mtDNA. Having a second independent allele which behaves similarly to *mss51-102* could provide additional verification of our *cis/trans* hypothesis for the action of Mss51. It would be particularly interesting if I could identify intragenic suppressors of *mss51-103*, and even more so if these suppressors contained either the A219D or N205D missense mutations identified as intragenic suppressors of *mss51-102*.

ACKNOWLEDGEMENTS

mtDNA preparation and Southern blots were performed by Christine Butler.

Table 5.5 – *S. cerevisiae* strains used in Chapter 5		
Strain	**Genotype**	**Reference**
a1#12	MATα, *leu2Δ, arg8Δ::URA3, ade2-101, kar1-1* [ρ+, *cox2Δ::ARG8m*]	This study
DAU1	MATa, *ade2, ura3-Δ* [ρ+]	(Costanzo and Fox, 1988)
NAB75 rho0	MATα, *ade2, arg8Δ::hisG, ura3Δ* [ρ0]	This study
NB40-33A	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, his3-ΔHindIII* [ρ+]	This study
XPM47 rho0	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ0]	This study
XPM76	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM78	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	(Perez-Martinez, 2003)
XPM89	MATα*, lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+]	This study
XPM171	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
XPM174	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	(Perez-Martinez, 2003)
ZV18a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, COX14::3xMYC* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV22a	MATα, *lys2, leu2-3,112, arg8::hisG, his3Δ::HindIII, ura3::kanMX3, mss51-103::3xHA, COX14::3xMYC*	This

	[ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	study
ZV25a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-103::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV27a	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV60	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, SUP* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV61	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, SUP* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV62	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, SUP* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV63	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-104::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV64	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-105::3xHA* [ρ+, *cox1Δ::ARG8m, cox2Δ::COX1c, COX2*]	This study
ZV65	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV66	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51Δ::LEU2* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV67	MATa, *leu2-3,112, arg8::hisG, ura3-52, MSS51* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV81	MATa/MATα, *lys2/LYS2, leu2-3,112/LEU2, arg8::hisG/arg8::hisG, ura3-52/ura3Δ, ade2/ADE2* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV82	MATa/MATα, *lys2/LYS2, leu2-3,112/LEU2, arg8::hisG/arg8::hisG, ura3-52/ura3Δ, ade2/ADE2, cox14Δ::URA3/COX14, MSS51/MSS51::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV83	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, COX14::3xMYC/COX14, mss51-102::3xHA/mss51-*	This study

	102::3xHA [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	
ZV84	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, cox14Δ::URA3/COX14::3xMYC, mss51-102::3xHA/mss51-102::3xHA* [ρ+, *COX1(1-512)::ARG8m*, ΔΣaI, ΔΣbI]	This study
ZV85	MATa/MATα, *lys2/LYS2, leu2-3,112/LEU2, arg8::hisG/arg8::hisG, ura3-52/ura3Δ, ade2/ADE2* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV86	MATa/MATα, *lys2/LYS2, leu2-3,112/LEU2, arg8::hisG/arg8::hisG, ura3-52/ura3Δ, ade2/ADE2, MSS51::3xHA/MSS51, cox14Δ::URA3/COX14* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV87	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV88	MATa/MATα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, cox14Δ::URA3/COX14::3xMYC, mss51-102::3xHA/mss51-102::3xHA* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV89	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-1* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV90	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-2* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV91	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-3* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV92	MATa, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-4* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV93	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-1/ARGMUT*	This study

	[ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	
ZV94	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-2/ARGMUT* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV95	Mata/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-3/ARGMUT* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV96	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-4/ARGMUT* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV97	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-1/argmut-2* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV98	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-1/argmut-3* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV99	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-1/argmut-4* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV100	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-2/argmut-3* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV101	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-2/argmut-4* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV102	MATa/Matα, *lys2/lys2, leu2-3,112/leu2-3,112, arg8::hisG/arg8::hisG, ura3-52/ura3-52, mss51-102::3xHA/mss51-102::3xHA, argmut-3/argmut-4* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study

ZV103	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-1* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV104	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-2* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study
ZV105	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-3* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	(Amiott, 2006)
ZV106	MATα, *lys2, leu2-3,112, arg8::hisG, ura3-52, mss51-102::3xHA, argmut-4* [ρ+, *cox1Δ::ARG8m, cox1::COX1::cox2d, COX2*]	This study

All strains are congenic or isogenic to D273-10B

Mitochondrial genotypes are shown in brackets. ΔΣaI, ΔΣb refers to an intronless mitochondria derived from CK520

c Ectopic insertion of the chimeric COX1 upstream of COX2

d Rearranged mitochondrial genome derived from ZV65

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